An investigation of tumour cell contamination of bone marrow and peripheral blood stem cell harvests in patients with AML

Andrew James Cunningham

Ph.D
University of Edinburgh

1998

Declaration

I confirm that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge the assistance of others who helped me during the course of this work.

Andrew Cunningham

For Jim and Corina

Lynn and Ellie

Acknowledgements

I would like to acknowledge the support of the following people without whose support this thesis would not have been possible.

Firstly I would like to like to thank my wife and daughter who lived with a stranger for 6 months, and my father and family for their unshaking belief in me.

I would like to thank my supervisors Dr Alastair Parker and Dr Robert Anthony for providing the project and funding to enable this work to be carried out, and their colleagues Dr Jenny Craig and Dr Steve Rogers for providing access to the clinical material.

My colleagues in the Haematology Department at the Royal Infirmary of Edinburgh Rachel McGregor, Nicola McKelvie who assisted with the antisense uptake into normal blood cells. Dr David Stirling who performed the conjugation of antisense to carboxylate beads and assisted with many aspects of DNA molecular biology. Also thanks to Professor Keith James, Dr Anton Alexandroff, Dr Jim McCormack, Dr Ray Dang, Dr Huw Roddie, Dr Helen Pearson, Mr Calum Scott, Ms Justine McIlroy, Mrs Helen Cameron, Ms Helen Taylor, Ms Kay Samuel, Ms Ursula Menzel, Ms Hannah Polson and Ms Anna Krassowska for their good natured and lively humour and support during this difficult period. I am grateful to Mrs Anita Ford and her staff of the Haematology Department at the Western General Hospital, Edinburgh, who performed the immunophenotyping for me and assisted with many aspects of acute leukaemia classification.

I would like to offer a sincere debt of thanks to Professor John Ansell and Dr Marc Turner without whose faith in me I would not be in a position to complete this thesis.

Abstract

Patients with acute myeloid leukaemia (AML) frequently relapse after chemotherapy-induced remission from their disease and effectivly become resistant to treatment. Cells from these patients may demonstrate the ability to grow autonomously in culture and express high levels of bcl-2 protein which confers resistance to chemotherapy-induced apoptosis and a poorer disease prognosis. Bcl-2 protein levels were therefore quantified in normal and AML cells. Autologous bone-marrow and peripheral-blood progenitor cell transplantation offer a treatment option but may be contaminated by tumour cells which contribute to disease relapse. Strategies have therefore been devised to purge tumour cells from the harvest to improve chances of patient recovery. One treatment option which may bypass resistance to chemotherapy-induced programmed-cell death mechanisms uses a class of drugs called antisense. These drugs have been shown to inhibit the translation of specific DNA sequences *in vitro* but have had limited clinical efficacy due to poor cellular uptake and targetting. Bcl-2 therefore offers a cellular target for antisense-mediated inhibition in AML to improve the efficacy of chemotherapy and patient survival. I have therefore studied the mechanisms of cellular uptake of antisense into cells of normal and leukaemic phenotype and its effectiveness at the induction of apoptosis in these cells. Antisense was found to be preferentially associated with monocytes, neutrophils and eosinophils with lower levels associated with lymphocytes and KG1-a (AML) cells. This uptake was found to be associated with expression of the adhesion molecule Mac-1 (CD11b-CD18). However antisense appeared to be sequestered into cytoplasmic vesicles and was predominantly functionally ineffective at reducing bcl-2 protein levels or in the induction of apoptosis. Antisense effects were also studied in the presence and absence of apoptotic stimuli from Cycloheximide, Actinomycin D or Cytosine arabinoside. There were no potentiating effects of bcl-2 antisense with either Cycloheximide or Actinomycin D at inducing apoptosis of AML cells. However there was a synergistic effect of antisense bcl-2 in the presence of Cytosine arabinoside, an agent used in the clinical treatment of AML. It was not clear whether effects of antisense could be attributed to true antisense-mediated or non-specific polyanionic effects mediated through integrin binding. Therefore antisense bcl-2 does not appear to be an effective therapy on its own for the generic treatment of AML but may function as a second-line adjunct therapy in resistant or relapsed cases of AML which fail to respond to conventional chemotherapy. AML cells expressing CD11b are associated with a poorer disease prognosis, are more likely to have elevated bcl-2 levels and resistance to therapy. It is these cells which are most likely to show a synergistic tumouricidal effect of bcl-2 antisense and Cytosine arabinoside. The role of antisense as a general agent for purging bone-marrow harvests of leukaemic cells however has yet to be established.

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Abbreviations

Ab	Antibody
Act D	Actinomycin D
Ag	Antigen
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
cAMP	cyclic Adenosine Mono-Phosphate
APML	Acute promyelocytic leukaemia
Ara C	Cytosine Arabinoside
AS	Antisense
ASI	Auto-Stimulatory Index
As_2O_3	Arsenic trioxide
ATP	Adenosine Tri-Phosphate
BMH	Bone Marrow Harvest
BSA	Bovine serum albumin
CD	Differentiation Cluster
CFU	Colony Forming Unit
CFU-GM	Colony Forming Unit Granulocyte-Macrophage
Channel number (#)	fluorescence intensity (linear scale)
Ci	Curie
Cl	Chlorine
CHX	Cycloheximide
CM	Conditioned Medium
CML	Chronic Myeloid Leukaemia
cv	co-efficient of variation
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo-Nucleic Acid
cDNA	complementary Deoxyribo-Nucleic Acid
DNAse	Deoxyribonuclease
DTT	Dithiothreitol (Cleland's Reagent)
EDTA	Ethylene diamine tetra-amino acetic acid
EGTA	Ethylene glycol tetra-amino acetic acid
EtOH	Ethanol
FACS	Fluorescence Activated Cell Scanning (Sorting)
FAM	Fluorescein Addition Monomer
FCS	Foetal-calf serum
FDA	Fluorescein diacetate
FITC	Fluorescein iso-thiocyanate

FL-1	green fluorescence
FL-2	red fluorescence
FL-3	orange fluorescence
FSC	Forward Scatter (size)
g	gravitational force
g	grams
kg	kilograms
mg	milligrams
µg	micrograms
ng	nanograms
pg	picograms
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
G-CSF	Granulocyte Colony Stimulating Factor
^3H	Tritiated
HBSS	Hanks Balanced Salts Solution
HCO_3	Bicarbonate
hrs	hours
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Minimal Dulbecco's Medium
IU	International Unit(s)
K	Potassium
l	litre
µl	microlitres
log	logarithmic (base 10)
M	Molar
µM	microMolar
nM	nanoMolar
Mg	Magnesium
min	minutes
MIP-1α	Macrophage Inhibitory Protein 1-α
ml	millilitre
MeOH	Methanol
MNC	Mononuclear Cells
MTT	(3-[4,5 dimethylthiazol-2-yl]2,5 diphenyl tetrazolium bromide; Thiazolyl blue

Na	Sodium
NCM	Non-Conditioned Medium
NH_4	Ammonium
NHL	Non-Hodgkin's Lymphoma
NRS	Normal Rabbit Serum
NMS	Normal Mouse Serum
oligo(mer)	oligo-deoxyribonucleotide
p	short arm of chromosome
%	percentage
PAB	PBS-azide-BSA
PAGE	Poly-Acrylamide Gel-Electrophoresis
PBMNC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBSCH	Peripheral Blood Stem Cell Harvest
PE	Phycoerythrin
PE_1	Plating Efficiency 1 (blast cell colony assay)
PE_2	Plating Efficiency 2 (blast cell colony assay)
PFA	para-formaldehyde
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PIPES	1,4-Piperazinediethane sulphonic acid
PMA	Phorbol 12 myristyl 13 acetate
PMT	photo-multiplier tube
q	long arm of chromosome
QBSF-51	Serum-free Medium
RBC	Red Blood Cell (Erythrocyte)
r-hu	recombinant human
RNA	Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
tRNA	transfer Ribonucleic Acid
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
s	seconds
SEM	Standard Error of the Mean
SSC	Side Scatter (granularity)
t()	chromosomal translocation
ter	terminal
TNF-α	Tumour Necrosis Factor-α

%w/v	grams weight per 100ml
%v/v	ml per 100ml
-ve	negative
+ve	positive
UV	ultraviolet
WBC	White Blood Cell (Leucocyte)
x	Mean

Introduction

Overview

Leukaemias are essentially tumours arising from blood cells. These leukaemic cells accumulate in the bone-marrow which eventually results in bone-marrow failure. The abnormal cells eventually spill out into the peripheral blood, causing an elevated white cell count. The consequences of bone marrow failure are anaemia (low red cell count), neutropenia (low neutrophil count resulting in inability to fight infections), thrombocytopenia (loss of platelets causing clotting disorders). In acute leukaemias, there may also be involvement of other organs (eg. spleen, liver, lymph nodes, CNS).

Classification of leukaemias:

Leukaemias are divided into acute or chronic forms.
When patients with acute leukaemia present they have by definition more than 50% blasts in their bone-marrow. The disease can be further classified on the basis of the primary cell type involved into acute myeloid leukaemia (AML) or acute lymphoblastic leukaemia (ALL). AML can be further sub-divided according to the French-American-British (FAB) system which assigns a classification number (M0 to M7) depending on the degree of cellular maturity.

When the total number of leukaemic blasts approaches 10^{12} the patient becomes ill from severe bone-marrow failure. Involvement of other organs may however not occur until the leukaemic cell population reaches 50% total blood cells or more.

Chemotherapy is used to induce a clinical remission where there are less than 5% blast cells present in the bone marrow. Disease relapse may only be confirmed by morphology using microscopy when the blast cells comprise more than 5% of all cells. At this level (5%) approximately 10^{8} leukaemic cells may still be present in the patient, however, providing other haematological parameters have returned to normal values this state corresponds to disease remission. Patients are said to relapse if more than this number of leukaemic cells are present following induction of complete remission with chemotherapy. Using sensitive cytogenetic, molecular biological or immunological techniques disease may still be detected at levels less than 5% although the blood and marrow appear histologically normal (Seivers et.al.1995). It is likely that this minimal residual disease (MRD) contributes to the frequent disease relapse seen in AML.

Origins

Ultimately the causes of leukaemia are not known although the acute leukaemias probably arise from selective proliferation of a single abnormal stem or progenitor cell which is able to form clonal daughter cells. In AML there is limited differentiation of the leukaemic blasts, but the cells are capable of further proliferation which causes the rapid replacement of the normal haemopoietic cells of the bone-marrow by leukaemic blasts. AML probably arises from a pluripotent stem cell or more mature myeloid progenitor. These myeloid progenitor cells are capable of differentiating into cells of the erythroid (red blood cells), granulocytic-monocytic (neutrophils, monocytes, eosinophils, basophils), and megakaryocytic (platelet) lineages. Lymphoid progenitors give rise to T and B cells. Typically AML blasts can be discriminated from ALL blasts by their expression of the cell surface proteins CD13 and CD33, do not express nuclear terminal deoxy-nucleotidyl transferase (TdT) and have T cell receptor genes in the germline configuration.

Patients treated with drugs for a primary (often so-called solid tumour eg. lung or breast cancer) cancer may develop leukaemia intitiated by the chemotherapy used to treat the primary disease, this is referred to as secondary AML. Of all the leukaemias, the acute leukaemias comprise more than 50%. AML may arise in any age group but is the most common form of (70-80%) adult acute leukaemias. Of childhood leukaemias only a minor fraction (10-15%) are AML with ALL being the most common form.

Therapy

For anaemic leukaemia patients with severe bone-marrow failure red blood-cell transfusions are given. Also since haemorrhage is an important early cause of death in many leukaemias regular platelet transfusions are given to correct the thrombocytopenia or during initial induction chemotherapy. This is a particular problem with patients with acute promyelocytic leukaemia (APML) who develop a severe disseminated intravascular coagulation (DIC) who may also be treated with fresh-frozen plasma. The severe reduction in neutrophils results in a reduced ability to fight infection. This may be treated with effective prophylactic use of antibiotics and growth-factors to stimulate production of neutrophilic cells.

Chemotherapy:

The primary aim of chemotherapy is to induce clinical remission (defined as less than 5% blast cells in the bone marrow) then to eliminate any remaining leukaemic cells by several additional consolidation courses of chemotherapy. Cyclical combinations of two, three or four drugs are given with treatment-free intervals to allow recovery of the marrow. Approximately 60-70% of adults with AML will attain complete remission after induction therapy, however remission rates in adults are inversely related to age.

The primary target for most cytotoxic drugs used in leukaemia chemotherapy are highly replicative cells. There are therefore non-specific effects on other highly replicative systems such as the gut, skin and hair follicles all of which contain regenerating compartments and are also able to recover following therapy. Cytotoxic combinations of three or more drugs are used to increase cytotoxic effects, overcome the potential for development of drug resistance and thus improve the likelihood of achieving remission

Induction therapy in AML normally consists of a combination of cytosine arabinoside, an anthracycline (daunorubicin) and either 6-thioguanine or etoposide. All AML sub-types (FAB M0-M7) are normally treated with similar drug combinations except for APML (M3) patients who receive *all-trans* retinoic acid in addition to chemotherapy to obtain remission. Two or three consolidation courses of intensive chemotherapy are usually given post-remission with similar drugs (Stone et.al.1993).

However remissions are not achieved in 10-20% of AML patients even following two courses of induction therapy. These high risk patients are normally selected for bone-marrow or peripheral-blood stem-cell transplants. In patients over 60 years of age such aggressive treatment is not normally an option because of the increased mortality so treatment options tend to be more limited (Hoffbrand et.al.1993).

Transplantation:

Transplantation of bone-marrow may be an option in patients under 45 years old with AML in first remission using HLA-matched bone-marrow from a donor (allogeneic bone-marrow transplantation (BMT). It may also be considered in patients who are in early stages of disease relapse or are in their second or subsequent remissions. Bone-marrow transplantation allows the functional recovery of the haematopoietic and immune systems following the myelo-ablative therapy used to kill the remaining leukaemic blasts.

There may be a beneficial side effect of this treatment if T cells from the donor marrow recognise the leukaemic patient cells as foreign and initiate a rejection reaction (graft-versus-host disease GVHD). This type of response may also result in a reduced risk of relapse by also rejecting the leukaemic blast cells in a graft-versus leukaemia (GVL) reaction. Bone-marrow donations from an identical twin (syngeneic marrow) or from the patient themselves (autologous marrow) carry little risk of this potentially fatal GVHD but also no potential for the beneficial (and potentially curative GVL). These types of transplant are normally carried out in first remission or after relapse and re-induction therapy (second remission). There is however the potential to transplant tumour cells with an autologous transplant which may require purging of the leukaemic blasts. Recently there has been a move to utilise stem cells (cells required to recover the haematopoietic system) which have been collected from peripheral blood rather than bone-marrow. These are refered to as peripheral blood progenitor cell transplants (PBPCT) and have been associated with a quicker recovery of normal blood function parameters and lower side effects than bone-marrow transplantation. This type of therapy is also cheaper and involves less morbidity and mortality to the patient as well.

The prognosis for remission induction and survival is determined by prognostic indicators such as immunophenotype, possession of certain cytogenetic abnormalities or growth characteristics of blast cells. Patients who are considered a good risk are given chemotherapy only; standard risk patients are treated with chemotherapy, and may be recommended for transplant; patients who are deemed poor risk are given transplant if a good donor is available, otherwise intensive chemotherapy is given. Patients with secondary AML tend not to respond to standard treatment and because of the toxic effects on the marrow from treatment of the initial disease most patients are unable to have an autologous stem cell transplant, however if they are otherwise suitable they may be eligible for allogeneic bone-marrow. Overall the chances of obtaining an initial remission with chemotherapy are between 50 and 80% dependent on age. Long-term survival is dependent on whether the patient is in a good, standard or poor risk category. Good risk patients have a 5 year survival of about 60%; for patients with standard risk disease it is much harder to predict the likely outcome

without a transplant (Hunter et.al.1993). Patients with poor risk disease have an extremely low chance of long-term survival without a successful bone-marrow or stem cell transplant.

Therefore in this thesis an alternative treatment option for patients in standard or poor risk categories was sought. The potential efficacy of one of the new promising molecular therapies was studied to look for an enhancement of tumour cell killing. This "antisense" therapy specifically targets bcl-2, a protein associated with the prolonged survival of many types of tumour cell including leukaemic cells. This drug can be administered on its own or in combination with standard chemotherapeutic regimens and should enhance their effectiveness at killing leukaemic cells.

Origins of Acute Myeloid Leukaemia (AML)

Many forms of AML are associated with specific chromosomal abnormalities including translocations where one part of a chromosome becomes aberrantly linked to part of another chromosome resulting in the production of disregulated protein expression or the formation of a novel fusion gene-product. Gene inversions, duplications or deletions may similarly occur. This has often lead to the functional characterisation of normal proteins involved in cell proliferation or differentiation.

Oncogenesis

Exposure of bone-marrow to ionising radiation can cause leukaemia through induction of chromosomal breaks, recombinations or point mutation activation of oncogenes, translocations and gene rearrangements may also frequently occur in leukaemias and lymphomas. For example gene rearrangements of the immunoglobulin (Ig) gene or T cell receptor (TCR) give rise to fusion genes. Translocations may also occur in other types of leukaemia and lymphomas eg. the Philadelphia translocation t(9; 22) in CML involving abl tyrosine-kinase; the t(15;17) translocation, involving the retinoic acid receptor, is associated with acute promyelocytic leukaemia (APML-M3); or the t(14;18) translocation, involving bcl-2 which is found in approx 85% of follicular lymphomas. Evidence that activated oncogenes are involved in leukaemogenesis is however somewhat indirect, though it has been possible to reproduce some leukaemic phenotypes in mice with putative oncogenes introduced into the germ-line. There is an increased risk of leukaemia associated with some hereditary diseases, particularly Down's syndrome (trisomy 21- where acute leukaemia especially ALL, may have an increased frequency). There is also an increased risk if family members have leukaekmia with a greater risk greater in identical twins. Existing haematological disease such as myeloproliferative diseases may also predispose to AML. Treatment of these conditions with chemotherapeutic drugs may also induce leukaemic progression through damage of oncogene DNA. This progresssion of leukaemic stem or early progenitor cells may also be associated with development of new chromosomal abnormalities.

Haematopoietic cells require growth-factors for survival and proliferation and changes in the function of receptors for these molecules are associated with leukaemia progression, though do not initiate the disease (Lang et.al.1990). Growth-factor receptors may have serine-threonine or tyrosine-kinase activity which induces phosphorylation of other signal transduction proteins including the receptor itself. Growth-factor receptors either directly, or more often through second-messenger signalling cascades, activate genes through transcription factors which bind DNA promoter sites and regulate the transcription of genes. The proteins coded for by these

genes often control the proliferation and differentiation of cells and are normally strictly regulated in a spatial and temporal fashion. Functional disregulation or deletion of these genes is thought to play an important role in leukaemic progression. Loss or functional inactivation of tumour suppressor genes such as p53 or retinoblastoma have also been described in CML in blast transformation, AML and MDS. Alteration of one allele may have a dominant effect if the mutated protein overrides the normal function of the protein (Sherr, 1996). For example tumour suppressor proteins such as p53 and the retinoblastoma protein act as control mechanisms which regulate the cell cycle and alterations in these proteins have fundamental effects on cell cycle progression in AML (Russell et.al.1995). Similarly gains of all or parts of chromosomes may result in amplification of genes and are common in the leukaemias and myelodysplasias and commonly include extra copies of chromosomes 8, 12, 19, 21 and losses affecting chromosomes 5, 6, 7, 11, 20 and Y (Hoffbrand et.al.1993).

There are also an important family of proteins whose role appears to be in the prevention of cell death in response to a number of potentially life-threatening stimuli. Cells deprived of growth factors or damaged cells may undergo a genetically programmed cell death known as apoptosis. The oncogene BCL-2 codes for a protein which inhibits cell death by apoptosis and elevated levels of bcl-2 protein are requently found in leukaemias and other tumours and contribute to resistance to chemotherapy (Hickman et.al.1994). The mechanism by which bcl-2 protein levels are regulated is at present unclear but is likely to be controlled by signals from growth-factors.

AML Classification

Morphology

The AML FAB subtypes are associated with different morphology and cytochemical staining and in many cases characteristic chromosomal changes and expression of surface proteins. Antibodies are helpful in the differentiation of ALL from AML and in the diagnosis of AML M6 or M7. The treatment and prognosis of each of the classifications are basically similar but there are some clinical differences as well as differences in treatment. However a study of 448 AML patients in first complete remission showed better disease free survival (DFS) with AML FAB 1, 2, 3 than M4 or 5. Sub-groups of AML patients also have poor prognosis; AML secondary to therapy; AML preceded by myelodysplastic syndrome; chromosomal abnormalities eg. deletions involving chromosomes 5, 7, trisomy 8, Philadelphia positive AML, or a white cell count greater than 100 $x10^9$ per litre (Geller, 1993).

In most cases clinical features and morphology can discriminate a diagnosis of AML from ALL. In ALL the blasts show no differentiation (with the exception of B-ALL) whereas in AML some evidence of limited differentiation to granulocytes or monocytes is usually seen. When the blasts are un-differentiated special tests are required to confirm the diagnosis and to classify and sub-divide the leukaemia. Cytochemistry eg. for myeloperoxidase may help to show granule development or non-specific esterases (NSE) for monocytic differentiation in AML. Immunological markers can also be used to distinguish AML from ALL, myeloid leukaemic cells are usually CD13+, CD33+ and TdT-. Occasionally a hybrid acute leukaemia can arise in which blast cells show features of both AML and ALL. These features may be on the same cell (bi-phenotypic) or on separate populations (bi-lineage). They include inappropriate expression of immunological markers eg. TdT+ AML or CD13+ ALL or inappropriate gene rearrangements and treatment is usually given on the basis of the dominant cell type.

AML accounts for 77% of the acute leukaemias (in adults 89%, in children 19%). These are broken down as follows M1 - 18%, M2 - 28%, M3 - 8%, M4 - 27%, M5 - 10%, M6 - 4%, M7 - 5% (Walker et.al.1994). Briefly AML M1, M2, and M3 show predominantly granulocytic differentiation and differ in the extent and nature of granulocytic maturation; M4 shows both granulocytic and monocytic differentiation; M5 has a predominantly monocytic differentiation and M6 predominantly erythroblastic differentiation; M7 is associated with leukaemic megakaryocytes.

The diagnosis of minimally differentiated leukaemia (M0) is made if myeloperoxidase

markers and megakaryocyte markers.

In myeloblastic leukaemia without maturation (M1) blasts show evidence of granulocytic differentiation with more than 3% of the blasts myeloperoxidase positive, maturation beyond this stage is not apparent (Knapp et.al.1994).

In patients with myeloblastic leukaemia with maturation (M2) blasts can be distinguished from M1 by cells maturating to, or beyond, the promyelocyte stage (myelocytes, metamyelocytes, and mature granulocytes may be found in varying proportions). The leukemic cells often have prominent nucleoli with varying amounts of cytoplasm, usually with many azurophilic granules. A specific chromosomal translocation (8;21) is frequently associated with this morphology. AML M2 has more than 30% myeloblasts with greater than 3% MPO+ with greater than 10% maturing granulocytes and less than 20% monocytic cells.

The t(15;17) translocation is associated with acute promyelocytic leukaemia (APML-M3) which involves translocation of the retinoic acid receptor. The majority of cells are abnormal promyelocytes, with a characteristic pattern of heavy granulation. A severe coagulation disorder - disseminated intravascular coagulation (DIC) is almost invariably associated with M3 AML sub-type. A small number of patients with APML have a version of this leukaemia known as M3 variant (M3V).

Myelomonocytic leukaemia (M4) cells have both granulocytic and monocytic differentiation in the bone-marrow and peripheral blood. M4 resembles M2 except that the proportion of promonocytes and monocytes exceeds 20% of the white blood cells. M4E variant has a variable (usually < 10%) number of abnormal eosinophils present. Most cases are associated with changes in chromosome 16 [inv(16)] and have a good prognosis.

Poorly differentiated monoblastic leukaemia (M5) is characterized by large blasts with several nucleoli. The large cytoplasm is basophilic and often pseudopodia are present. Monoblasts, promonocytes, and monocytes are found; with more monocytes in the peripheral blood than the bone-marrow, in which the predominant cell is the promonocyte. Infiltration of other tissues are associated with this phenotype particularly of the skin and gums, are common in patients this morphologic subtype.

Erythroleukaemia (M6) involving is characterised by an excess of erthyhroblasts. The blast cell count (excluding erythroblasts) exceeds 30% of nucleated cells in the bone-marrow though the percentage of myeloblasts and promyelocytes with erythropoietic changes can be variable.

M7 (megakaryoblastic) leukaemia is often accompanied by fibrosis of the bone-marrow. Blasts can either resemble immature megakaryocytes or be undifferentiated resembling lymphoblasts. The diagnosis can be confirmed by ultrastructural demonstration of platelet peroxidase or detection of platelet antigens CD41 or CD61 (Hirsch-Ginsberg et.al.1993).

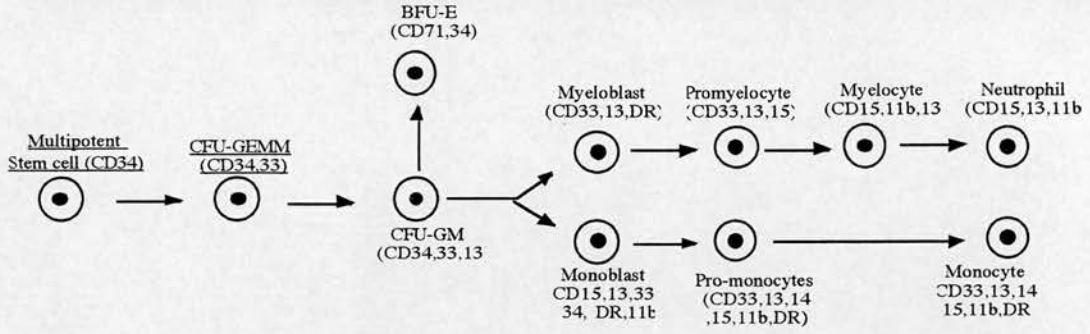
Cytogenetics

Certain chromosome changes are typical of different AML sub-types and can be useful in the diagnosis and monitoring of residual disease (MRD) during and following therapy. Chromosome changes may carry a prognostic significance in AML, with the most frequent changes being trisomy 8 or monosomy 7 (Lowenberg et.al.1993). In children or young adults there are more balanced translocations, however in secondary AML or elderly patients there are usually numerical and unbalanced changes. The t(8;21) translocation, involving the AML-1 and ETO gene fusion, is the most common, found in 10% AML associated with M2. A complete remission (CR) rate of 80% and disease-free survival (DFS) of 67% is associated with this form of the disease. The t(3;21) translocation also involves the AML-1 gene and the EVI-1 gene; M2 with t(8;21) which involves the AML-1 gene (which has an ATP binding domain). The t(15;17) is associated with most cases in APML and results in severe coagulation disorder but is otherwise associated with a better prognosis (Grimwade et.al.1997). APML t(15;17) results in a fusion gene between PML (zinc finger protein) with the retinoic acid receptor. The inv(16) chromosomal inversion accounts for 16% of cytogenetic abnormalities in AML and is mainly associated with the M4E variant but also M2 and M4, 5, MDS and CML in blast crisis. Deletions of chromosome 5q are associated with therapy-related AML/MDS (secondary disease) and confers a poorer prognosis. The receptors for growth-factor receptors IL-3, 4, 5, 9, GM-CSF, CSF-1 receptor, CD14, EGR-1 (zinc finger transcription factor), IRF-1 (interferon response factor) are all found on 5q31.(De Greef et.al.1996). Other less common changes seen are trisomy 4 in M2 or M4 (also trisomy 8 or 22), also t(8;16), inv(16) in M4; trisomy 8 is also seen in M5 as is t(8;16) (Walker et.al.1994).

Immunological classification.

Widespread application of flow cytometry using fluorescent-tagged monoclonal antibodies has allowed routine immunological classification of acute leukaemias. For example antibodies to myeloperoxidase, CD33 and CD13 identify virtually all myeloid leukaemias.

Immunologic markers on normal haematopoietic cells normally forms a lineage-restricted and differentiation-specific sequence (**Figure 1**).



(**Figure 1**) (Freedman et.al.1993)

In AML many of these antigens are also expressed but either in the incorrect sequence or with co-expression of lymphoid markers. There is unfortunately no single marker to identify the leukaemias. There does not appear to be direct correlation between immunophenotype and prognosis though some individual markers are associated with a good or poor response rates.

Multi-parameter flow cytomety (which utilises 3 or more colours in combination with light scatter characteristics) has found, for example TdT and CD7 co-expression with the normal myeloid markers CD13 and CD33. No normal tissues have been found to express these markers, however this is limited to only 25% AML patients (Campana et.al.1995; Campana, 1993). Terstappen et.al.1992 have described patterns of expression of normal cell-surface antigens in AML, expression of non-myeloid antigens, asynchronous expression of myeloid antigens or expression on immature cells, overexpression of myeloid antigens, absence of myeloid antigens in AML.

Under the FAB system the presence of more than 3% myeloperoxidase (MPO) positive blasts confirms a diagnosis of AML. Using monoclonal antibodies on permeabilised cells it is possible to detect MPO in the majority of AML samples and CD34 positive cells. APML (M3) and the majority of CD14 positive AML (monocytic) do not however express the CD34 marker. CD13 and CD14 are markers associated with a low complete remission rate, but a favourable outcome is associated in patients with CD15 associated with M5 and M4. CD34 is associated with M0 and M1 and M5a but not M3 or M5b (Campos et.al.1989).

The majority of AML express stem cell factor CD117 (ckit receptor) on early blasts, though some are CD34-, CD117-. CD15 (a later myeloid antigen) is associated with favourable outcome though it can be lacking on M3 promyelocytes (Knapp et.al.1994). CD4 is found on up to 75% adult AML, CD7 is expressed in 10% AML (Hirsch-Ginsberg et.al.1993). The CD34 marker is expressed on up to 70% AML patients and is normally found on haematopoietic progenitors, high endothelial venules (HEV), vascular endothelium and some fibroblasts and may play a role in progenitor cell adhesion to bone-marrow stroma (Steen et.al.1996).

M0 accounts for 5-10% of all AML cases. M0 AML patient blasts are 70-80% CD13, and CD33 positive there is also consistent expression of CD34 and HLA-DR and antibody-detected myelo-peroxidase (MPO) (Castoldi et.al.1996). These cells are normally CD7 and TdT negative. No chromosomal abnormalities are specific for this type of leukaemia, however -7/7q-, +13. -5/5q- may be present. M0 can be diagnosed with certainty using antibodies to myeloid associated antigens CD13, 14, 15, 33, 34, and HLA-DR. AML M0 have a poor prognosis if positive for CD34, CD7, HLA-DR and TdT. M0 are normally found to be negative for CD22, 3, 13, 19, 10, 37, 2, 33, 14, 61 and glycophorin A

M1 can be positive for CD11b, however CD14 and 15 are usually negative.
M1 and M2 can express CD34, 33, 13, HLA-DR and 20% patients express CD14.

CD15 is associated with M2 though karyotyping gives the definitive diagnosis.

APML M3 has strong MPO+ cells and non-specific esterases (NSE) and is associated with the t(15;17) karyotype. M3 are CD34 and HLA-DR negative as are 20% of M1 and M2 and 10% of M4 and M5). The presence of NK cell / myeloid markers in acute leukaemia may appear to be M3 by morphology, but do not demonstrate any detectable PML-RAR fusion transcripts (De Greef et.al.1996).

M4 myelomonocytic AML cells are MPO+, non-specific esterase (NSE) positive and

M4 myelomonocytic AML cells are MPO+, non-specific esterase (NSE) positive and more than 20% cells are pro-monocytes. The (inv16) M4 variant has an increased level of eosinophils. M4 and M5 can express CD13, 33, 11c, 15, 14, 36, 11b, possibly cytoplasmic lysozyme, CD4, 7,19, or the NK markers CD16 or 56.

M5 AML (monocytic leukaemia) has diffuse NSE positivity with less than 3% MPO positive cells.

M6 AML (erythroleukaemia) has similar morphology to M1, 2, or 4 blasts but with the presence of dysplastic erythroid precursors. M6 can express glycophorin A, CD71 (transferrin receptor), and may also express CD36, CD7, 15, HLA-DR.

CD41, 42, 61 antigens are predominantly found on AML M6 and 7.

M7 are known to express CD61 (platelet glycoprotein IIIa), von Willebrand antigen, CD41 (Freedman et.al.1993).

Generally speaking patients with a worse complete remission rate and survival include CD13, CD33, CD14 , CD11b, HLA-DR, CD34, CD9, MDR and bcl-2 whilst only CD15 indicates a better complete remission and survival (Sanz et.al.1996).

Therapy

Treatment for AML is normally in two distinct phases:

a) induction therapy used to achieve disease remission and

b) consolidation therapy to prevent further disease relapse.

Consolidation therapy appears to be most effective when given either immediately after remission or can be delayed for several months.

Induction therapy usually produces marrow aplasia and together with the leukaemia-induced bone-marrow failure patients therefore require support including red cell and platelet transfusions as well as treatment for infections. Colony-stimulating factors eg. granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) are currently used to increase the number and activation state of neutrophils. These growth-factors are normally administered following induction therapy resulting in a shorter neutropenic period and reduced incidence of infection. Drugs such as cytosine arabinoside are proliferation (S-phase) specific and as such affect replicating cells, however some leukaemic cells have a long dividing time and may escape chemotherapy. Growth-factor mobilisation of non-proliferating leukaemic pogenitors into cycle may augment S-phase specific drugs (Schiller, 1991). Studies have noted that simultaneous addition of cytosine arabinoside (AraC) and GM-

CSF resulted in greater cell death (Stone et.al.1993). Mobilisation of stem or progenitor cells with G-CSF or GM-CSF may also lead to proliferation of leukaemic clones and cause disease relapse. Growth-factors may also induce differentiation eg. in the treatment of APML retinoic acid is routinely used to induce terminal differentiation followed by cell death, M-CSF has also been used to induce terminal differentiation in leukaemic blasts (Bernstein, 1993).

Unfortunately the majority of AML patients respond only transiently to therapy and ultimately die of their disease. Following induction therapy using anthracycline (daunorubicin) and cytosine arabinoside (AraC) 70-80% AML patients achieve complete remission but many subsequently relapse and die resulting in a poor (20-60%) 5-year survival rate (Schiller, 1991). Treatment options for these relapsed or refractory patients include chemotherapy (high dose cytosine arabinoside, idarubicin, etoposide), autologous or allogeneic bone-marrow or peripheral blood progenitor transplant, or combined chemotherapy plus growth-factors. High dose cytosine arabinoside is associated with 40% complete remission in patients with relapsed, refractory or secondary AML (Stone et.al.1993). Most AML patients in first remission are treated with AraC consolidation therapy alone, or followed by allogeneic or autologous bone-marrow or peripheral blood progenitor cell transplantation or autologous peripheral blood stem cell transplantation. Allogeneic BMT allows the potential for a curative graft-versus-leukaemia effect, however post-transplant survival is similar to survival following remission induction chemotherapy due to transplant-associated mortality. Allogeneic transplantation however remains the only potential for a cure after disease relapse. The efficacy of using autologous transplantation following purging is currently being studied.

Drug resistance

Leukaemic cells often develop resistance to chemotherapy following treatment, probably due to the selective expansion of cells with an underlying resistance mechanism eg. presence of the multi-drug resistance protein (MDR-1) (Sonneveld, 1996). Resistant cells express high levels of p-glycoprotein (MDR-1) which is an ATP-dependent drug efflux pump. MDR can however be blocked by cyclosporin A or verapamil. MDR-1 is frequently found in cases of *de novo* AML (19-75% of untreated cases). Patients with refractory or relapsed AML express MDR-1 (which can be detected by flow cytometry) more frequently . Drugs which block MDR result in a higher intracellular concentration of cytotoxic drugs, though this may also affect normal CD34 positive progenitors which normally express p-glycoprotein.

Other mechanisms of drug inactivation include glutathione-S-transferases which inactivate drugs by the addition of GSH. For cytotoxic effects cytosine arabinoside requres to be phosphorylated to Ara-CTP which then competes with dCTP for incorporation into DNA by polymerases. After incorporation into DNA Ara-CTP acts as a chain terminator (Sonneveld, 1996). Resistance to retinoic acid (ATRA) can develop through a lowering of plasma levels of ATRA through a cytochrome p450-mediated mechanism. Treatment with 9-cis retinoic acid however is able to overcome this plasma clearance (Fenaux et.al.1996).

Minimal and Residual Disease (MRD)

Following induction of disease remission with chemotherapy patients may have less than 5% blasts (detected by light microscopy) together with recovery of normal hematopoietic parameters. Approximately 75% of AML patients achieve this type of remission though the majority subsequently relapse, it is unfortunately difficult to predict who will relapse. Patients in clinical remission with 5% tumour cells represents a tumour burden of between 10^8-10^{10} leukaemic cells and this "minimal residual disease" (MRD) is not detectable by conventional means (Stone et.al.1993). More sensitive methods for detection of MRD include cytogenetics, FISH, colony growth, PCR and multi-parameter flow cytometry.

Cytogenetics is able to detect the presence of 1 tumour cell in 10 normal cells, fluorescence *in situ* hybridisation (FISH) detects 1 cell in 10-100. Southern blotting using labelled DNA probes can detect 1 tumour cell in 100-1000 normal cells, flow cytometric antibody detection1 cell in 40,000 (Dwenger et.al.1996). Detection of chromosomal translocations are the most useful method for monitoring AML since they are tumour-specific markers detectable by the polymerase chain reaction (PCR). Positive PCR results does not always mean imminent relapse as the patient can be in complete remission (CR), however increasing PCR positivity may prelude a imminent relapse. Monitoring MRD in AML does not seem to be relevant for changing clinical treatment. Contrastingly APML (M3) with t(15;17) positivity is associated with relapse. Presumably these resistant MRD cells are non-dividing or slow-cycling cells since most chemotherapy acts upon cells in S-phase. Leukaemic cells in the bone-marrow may however not show the nuclear proliferation antigen. Other strategies such as the use of growth-factors to mobilise cells into cycle may be effective eg. CML progenitors can be induced to differentiate into CML dendritic cells (using, TNF-α, IL-4, GM-CSF) which are able elicit an anti-CML tumour response (Dwenger et.al.1996).

Molecular Techniques

Conventional cytogenetic karyotypic analyses detect only residual dividing leukaemic cells in metaphase. Fluorescent *in situ* hybridisation (FISH) techniques can be used to detect numerical chromosomal changes of cells in interphase. Translocated or mutated oncogenes may be detected by polymerase chain reaction (PCR) to look for tumour-specific gene markers. These molecular tests using PCR may offer a sensitivity of detection of one tumour cell in 10^5 normal cells. This however must be interpreted with caution because of the potential for changes in genotype. Clonal evolution may arise so a negative result may be misleading during disease progression and there may

also be patchy distribution of MRD within marrow or blood. For example disappearance of bcr-abl transcripts in B-ALL after BMT may subsequently reappear with disease relapse (Nizet et.al.1991). PCR can be used to detect chromosomal translocations in AML eg. t(15;17); t(8;21); inv(16); t(9;22), t(6;9) or RT-PCR to detect APML M3 (Campana et.al.1995; Langlands et.al.1993).

Patients with APML are treated with ATRA to induce differentiation of the leukaemic clone and results in remission. Molecular techniques always detect residual disease in these patients, RT-PCR offers the most rapid diagnosis and all patients positive at the end of therapy subsequently relapsed. The t(8;21) translocation, associated with 20% of M2 AML cases, causes a fusion gene to be formed between AML-1 and ETO genes. RT-PCR again offers the most rapid diagnosis. The inv(16) mutation is associated with acute myelomonocytic leukaemia with excess of eosinophils. A fusion gene consisting of CBFβ (core binding factor beta) and MYH-II (myosin heavy chain) again can be detected by molecular techniques using RT-PCR for diagnosis. The t(6;9) translocation seen infrequently in AML can also be detected by RT-PCR of the fusion gene of DEK and CAN (Morgan, 1994).

Southern blots only detect 1-5% abnormal cells in a population of normal cells. PCR using the T cell receptor gene rearrangements detects one clonal cell in 10^3 normal polyclonal cells. However the sensitivity can be increased using allele specific primers to detect one clonal cell in 10^5-10^6 normal cells. Patients maintaining CR showed a decline in PCR detectable abnormal cells, but maintained steady levels or even rose when patients relapsed (Nizet et.al.1991). T cell gene rearrangements are present in 10-40% of AML cases. PCR sensitivity using T cell receptor (TCR) delta gene or t(14;18), or IgH gives a sensitivity of one tumour cell in 10^4-10^5 normal cells (Langlands et.al.1992). Again using allele (patient) specific primers PCR can detect point mutations in N and K-ras or p53 found in 25-30% AML patients (Russell et.al.1995).

Flow cytometry in MRD

There is unfortunatley no single immunologic marker which is suitable for detection or classification of AML. Normal phenotypic markers are expressed and it may only be the combination of markers or their relative levels which distinguish the leukaemic from normal cells (**Figure 1**). Immunologic markers detected by flow cytometry allow one cell in 10^4 to be detected. For example flow cytometry using a combination of immunological markers to detect "leukaemia-specific" combinations of antigens eg. TdT+ myeloid cells are present in 75% cases of AML (Campana et.al.1995). Single immunologic markers are therefore not appropriate for MRD detection. Two, three or even four colour analysis help identify lineage infidelity (eg. lymphoid antigens on myeloid blasts), asynchronous antigen expression (**Table 1**). For example, 40-70% AML patients have antigen expression which allow 10^4 tumour cells detection by immunophenotyping (Castoldi et.al.1996).

CD	13/33	11b	15	14/36	GlycA	41/61	DR	TdT	34	9
FAB										
M0	+	-	+/-	-	-	-	+	+	+	-
M1	+	+/-	-	-	-	-	+	+	+	-
M2	+	+/-	+	-	-	-	+	-	-	-
M3	+	-	+/-	-	-	-	-	-	-	+
M4	+	+/-	+	+	-	-	+	+/-	-	-
M5	+	+/-	+/-	+	-	-	+	+/-	-	-
M6	+/-	+/-	-	+	+	-	+/-	-	-	-
M7	+/-	-	-	+	-	+	+	-	+/-	+

(**Table 1**) Possible combinations of cell surface marker expression in AML.

Four patterns of aberrant expression of normal cell surface proteins:

a) expression of non-myeloid antigens eg CD2, CD5, CD7 on myeloid blasts.

b) asynchronous expression of myeloid antigens eg. co-expression of CD34 and CD15

or CD16.
c) over-expression of myeloid antigens eg. increased CD34 and CD14 expression on neutrophils.
d) absence of myeloid antigens eg. lack of CD33 expression or co-expression of CD11b and CD15 (Terstappen et.al.1992).

45-70% of AML patients express CD34 (Nimgaonkar et.al.1996; Brito-Babapulle et.al.1990) though AML M1, 2, 3 do not show any differences in CD34 epitope expression, though normal hematopoietic CD34 positive cells express class III epitopes rather than class I or II (Steen et.al.1996).

Studies using markers for solid tumours have confirmed the presence of tumour cells in peripheral blood, bone-marrow and apheresis products (Volpi et.al.1994; Brenner et.al.1993). Using anti-cytokeratin and an anti-CD45 antibody to detect breast cancer cells it was found that tumour content (per CD45+ mononuclear cell) was greater in bone-marrow than peripheral blood or apheresis products. Cytokeratin however only reacts with approximately 80% breast cancer cells. This detection method allows the discovery of 1 tumour cell in 200,000 mononuclear cells (Clarkson, 1993). The sensitivity of the methodology can be increased by using 4-colour immuno-fluorescence in combination with immuno-magnetic cell-sorting where it is possible to detect a theoretical one tumour cell in 10^7 normal cells. Cytokeratin-19 is however also found in normal individuals without breast cancer (using nested PCR) (Pantel, 1996).

Colony assays in MRD

Clonogenic assays have indicated that the growth of AML blasts are derived from leukaemic progenitors (Clarkson et.al.1970; McCulloch, 1983). Some leukaemic progenitors have the capacity for self-renewal (Buick et.al.1979) however with restricted differentiation potential (Castoldi et.al.1996).

The first culture assays for leukaemic blast cells were modifications of existing culture methods for normal haematopoietic progenitors (Senn et.al.1967; Pike et.al.1970). These assays used an agar bi-layer system with a feeder layer of normal leucocytes to provide colony-stimulating factors known to be essential for the proliferation of the normal cells (Robinson et.al.1971). For detection of AML standard haematopoietic colony assays were relatively inefficient with poor yield, clusters (<40 cells) rather than colonies (>40 cells) tended to form and cells from only 30% of patients formed proper colonies (Spitzer et.al.1976). Growth of leukaemic blasts was found to be predominantly growth-factor dependent and used methyl-cellulose for the mechanical support for the colonies, and conditioned medium from cell lines as the source of

colony-stimulating factors (Buick et.al.1977). Colonies were found to be present in 81% of patients in remission and patients with relapse therefore it was not felt to be a reliable indicator of relapse although the sensitivity of these colonies to 4-hydro-peroxycyclophophamide (4-HC) was (Campana et.al.1995). There is also the problem that some AML blasts with the potential to cause clinical relapse may fail to grow in culture systems (Seivers et.al.1995).

Colony assays are able to detect between 0.01 to 1% of colony forming cells. Up to 70% of AML blasts have been shown to exhibit partial or total autonomous growth in *in vitro* blast cell colony assays. There was a correlation between the ability of AML blasts to produce GM-CSF and IL-1β and the capability to grow autonomously *in vitro* (Bradbury et.al.1997; Reilly et.al.1989a). Blasts with the highest levels of GM-CSF were able to grow autonomously. AML blasts have been shown to produce GM-CSF, G-CSF, IL-1, IL-6, and TNF-α.

IL-1 and GM-CSF have been shown to act in an autocrine fashion, with IL-1 regulating levels of GM-CSF secretion which in turn causes blast cells to proliferate. Four patterns of colony formation were described and related to disease prognosis. Group 1 patients - showed no growth in culture: group 2 patients grew only in the presence of exogenously added colony-stimulating factoirs; group 3 patients were partially autonomous but showed enhanced growth upon addition of growth factors and finally group 4 patients who exhibited fully autonomous growth (Reilly et.al.1989a). Of a study of 114 patients with AML 37 were low proliferators, 39 intermediate and 38 showed a high proliferative potential. A 36% 3 year survival was associated with low proliferation rates (68% CR rate and 49% DFS); a 3% 3 year survival with high proliferation rates (39% CR rate and 11% DFS). AML M4 and M5 were shown to have a greater proliferative capacity than M1 and M2. There was no correlation of proliferation with age, sex, white blood cell (WBC) count or percentage blasts in the marrow though trisomy 8 was associated with poor CR (Lowenberg et.al.1993).

Purging

Myeloablative chemotherapy followed by allogeneic bone-marrow is considered curative for AML. This procedure can be considered for patients in first remission post chemotherapy. However only 10-20% patients will be eligible to receive allogeneic transplants because of age or lack of an available donor. Autologous transplants may also be considered but may harbour tumour (Hammert et.al.1997). Peripheral blood progenitor-cell (PBPC) transplants may be the only option when there is a hypocellular marrow (caused by prior therapy) or tumour involvement in the marrow, or lack of donors (Sharp et.al.1992). Combination chemotherapy can induce a remission on up to 80% AML, however more than 50% relapse, whilst salvage therapy may re-induce a second CR of 4 months. Autologous BMT (ABMT) for AML in first remission gives a DFS of 34-57%. Relapse is the major cause of failure of ABMT.

A series of elegant studies have shown the potential for transplanting tumour cells within autologous bone-marrow or PBPC grafts to contribute to disease recurrence. The St Jude's group (Rill et.al.1994; Brenner et.al.1993) labelled a portion of the graft with a neomycin resistance gene and showed that in 3 out of 4 relapse cases some of the patients' cells contained the marker. AML, CML, ALL, lymphoma, muliple myeloma and breast cancer, neuroblastoma have all been studied with gene marking and transplanted cells shown to contribute to disease relapse. For example the neomycin resistance gene was expressed found in CD34+, CD56+ leukaemic AMLs after relapse, markers which both were expressed on the original tumour. In normal transduced cells levels of the gene expression were greatest in myeloid >T cells > B cells (Heslop et.al.1995; Rill et.al.1994). The neomycin resistance gene was detected either by PCR on sorted blast cells or on resistant colonies grown on G418 (neomycin analogue) (Brenner et.al.1993).

The clinical value of purging has not been established by clinical trials (due to a lack of trials) though several studies suggest a contribution to relapse from non-purged grafts. However 20-70% breast cancer patients undergoing high dose chemotherapy have been shown to have tumour cells in otherwise histologically normal bone-marrow (Kvalheim et.al.1996).

CD34+ cells selected by immunomagnetic beads gives a 2-4 log reduction of tumour cells. Using 2 cycles of depletion gives up to 6 log reduction of tumour cells with 30-40% bone-marrow progenitor loss. Immunobeads can give a 5 log reduction, complement lysis 1-2 log reduction, immunotoxins 1 log reduction. Leukapheresis products frequently contain 2-4 x 10^{10} nucleated cells. CD34 immunomagnetic enrichment in breast cancer however only gives 2-4 log reduction of tumour (Kvalheim et.al.1996). Nevertheless CD34 positive cell selection is not possible in AML since up

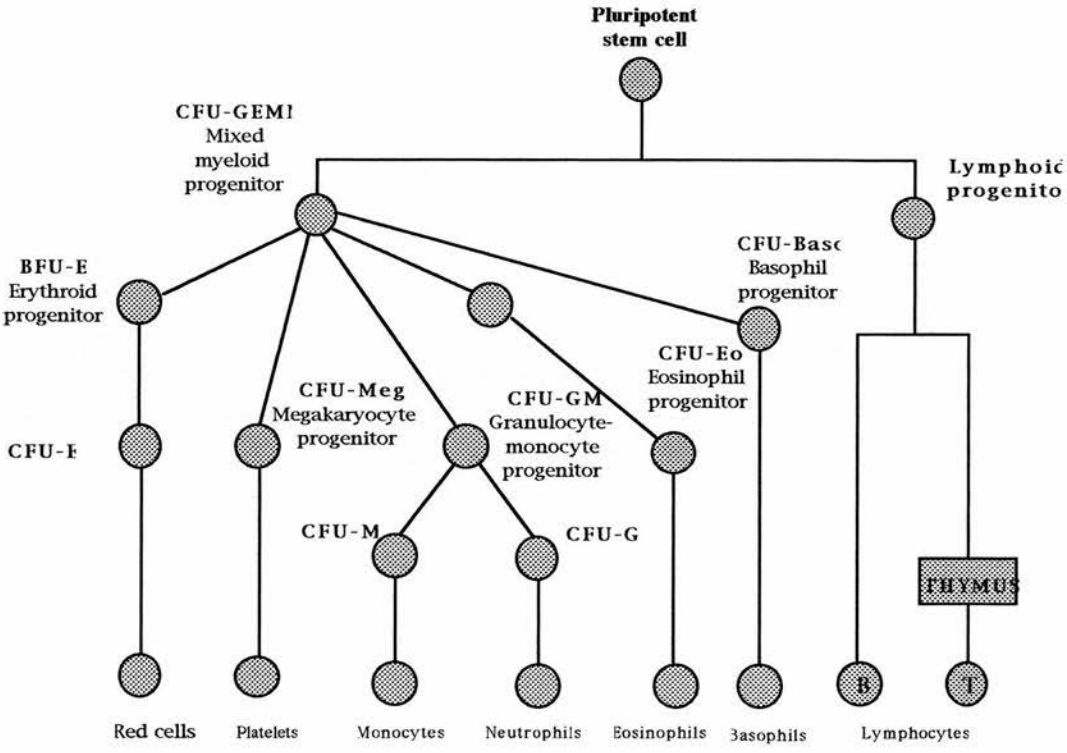
to 70% of AML cases will express CD34 (Nimgaonkar et.al.1996; Brito-Babapulle et.al.1990). It is possible however to get up to a 7 log reduction of AML blast cells and 1-4 log reduction in CD15 positive cells without loss of progenitors using a combination of CD34 selection and CD15 depletion. More than 95% AMLs expressed CD15 following neuraminidase treatment to reveal the epitopes and 45-70% AML express CD34 (Nimgaonkar et.al.1996). Using immunobeads it is possible to see a sensitivity of 2 cells in 10^7 mononuclear cells by selecting cells with greater than 3 beads per cell (Pantel et.al.1996).

Crypreservation has been shown to provide an element of tumour cell purging in AML though the reasons for this are unclear (Allieri et.al.1991) but may be related to blast cell differentiation caused by the cryopreservative DMSO.

It is may be however that complete tumour ablation may not be required, simply reducing the numbers to a manageable level may be enough. Patients with ALL who have less than 10^5 tumour cells have remained in remission (Dwenger et.al.1996). Animal studies have shown that a 3-4 log reduction in tumour burden can result in a curative transplant (Hammert et.al.1997). Whether this represents a manageable level for establishment of an anti-tumour response is unclear.

Stem cells and Haematopoiesis

Pluripotent stem cells are capable of self-renewal and respond to haematopoietic growth-factors by increasing the production of required lineages. Pluripotent stem cells resemble lymphocytes in terms of their size and granularity they are also wholly contained within the CD34+, Lin-,SCF+ cell compartment (Sutherland et.al.1994). A more mature common progenitor cell appears to give rise to a number of cell lineages including the erythroid, granulocytic, monocytic and megkaryocytic whilst another gives rise to cells of the lymphoid lineage (**Figure 2**). The earliest myeloid progenitor, called the CFU-GEMM (colony forming unit granulocyte-erythroid-monoctye-megakaryocyte), and lymphoid progenitors form in the bone-marrow which supports both stem cell growth and differentiation.



(Figure 2)

This figure demonstrates the normal hierarchical development of cells in the bone marrow and their subsequent maturation to functional haematopoietic cells.

The stem cells comprise only a very small percentage (less than 0.1%) of nucleated cells in the bone-marrow. Historically the CFU-GM has been the standard by which stem cells for clinical transplantation were enumerated. On average a patient required 7-10 leukaphereses to obtain enough CFU-GM per kg to ensure engraftment following peripheral blood progenitor cell (PBPC) transplantation (more than 3 x 10^5 CFU-GM per kg). Recently recombinant growth factors G-CSF or GM-CSF plus chemotherapy has been used to mobilise up to 2-300 more progenitors than normally circulate in peripheral blood and allows more sucessful engraftment (Shea et.al.1992).

Transplantation

Chemotherapy is the main form of treatment in leukaemia and in some cases radiotherapy may also be used. Unfortunately both anti-cancer drugs and radiotherapy also cause severe damage to normal cells in the bone-marrow. Spacing out the treatment over several months minimises the damage and allows recovery of normal marrow function. The most common reason for transplantation is to treat a leukaemia which has either not responded to therapy or has relapsed following initial therapy.

Allogeneic bone-marrow transplants may be useful in high-risk groups eg. chemoresistant AML. Allogeneic BMT are age-restricted (to less than 55 years) and compatibility of the HLA-donor. There may be as high as 50% relapse rate following allogeneic BM or PBPC transplants but the potential for for a graft-versus-leukaemia (GVL) effect makes it the treatment of choice. T cell depletion increases the relapse rate but decreases the GVHD and GVL reaction (Schiller, 1991).

Autologous Transplant

Some patients may be able to use their own autologous marrow or peripheral blood stem cells. This can be used for patients with CML, AML, lymphoma and myeloma and solid tumours including breast and testicular cancer. Autologous transplants may be offered to a large proportion of patients who are harvested in complete remission. There is a low risk of GVHD but no potential for a GVL and there is always the possibility of transplanting tumour, although various purging strategies have been tried, including 4-hydroperoxycyclophosphamide (4-HC) or immunologic purging (Rubin et.al.1994). Autologous marrow is harvested in complete remission at a time of minimal residual disease and then purged to remove tumour cells by pharmacologic or immunologic methods. However 4-HC is non-specific and also affects normal hemopoietic precursors.

It is possible to get a 1-2 log purging using antibody but there may be 10^6-10^9 tumour

cells in a complete remission marrow leaving 10^4-10^7 tumour cells. Purging may either destroy normal marrow function or provide incomplete tumour cell depletion and is therefore not ideal.

Sources of Stem Cells

Stem cells can be obtained from bone-marrow, the peripheral circulation or from the umbilical cord blood. Until recently bone-marrow had been the only source of stem cells for transplantation. Marrow can be frozen and stored for months or even years. This is often done for patients with chronic myeloid leukaemia or lymphomas. Storing marrow taken during remission may offer a therapeutic option if the leukaemia should return and may also reduce the tumour cell contamination (Allieri et.al.1991).

There are normally very small numbers of stem cells circulating in the blood known as peripheral blood progenitor cells (PBPC). Normally there are insufficient numbers of these to harvest from the blood though it is possible to recruit progenitor cells from the bone-marrow into the peripheral blood by using growth-factors on their own or in combination with a small dose of chemotherapy. GM-CSF has been shown to increase the numbers of CFU-GM in peripheral blood 60 fold following chemotherapy (Socinski et.al.1988). Patients who receive peripheral blood progenitor cell transplants spend a shorter time in hospital and have fewer complications because their neutrophil and platelet counts return to normal more rapidly. Autologous stem cells have the potential for contamination with tumour cells which may contribute to disease relapse. It may be possible to remove, or to reduce the numbers of leukaemia cells by purging techniques using antibodies to isolate stem cells then return them to the patient. A similar approach is to use antibodies against the leukaemia cells to deplete them from the marrow. Both methods can be used on the same harvest.

The ideal donor is an identical twin because tissue types will be identical to the patient. A transplant between identical twins is called a syngeneic transplant. There are no problems with rejection or GVHD though the relapse rate is higher than with allogeneic grafts. The next most suitable donor is a matched sibling. The remainder of patients require a matched-unrelated donor (MUD). This however requires close tissue-type cross-matching and requires continual immunosuppression. The closeness of a match needed for a good result depends on which of the human leucocyte antigens (HLA) are mismatched.

Leukaemic patients who are considered good risk are not normally given a transplant as treatment can be more dangerous than beneficial. Autologous and allogeneic transplants can both be used for the treatment of acute leukaemia even though the

but benefit from being free of leukaemia cells which could be re-infused at the time of transplant, and may also exert a GVL which is potentially curative.

AML patients with a good risk category of disease have an excellent chance of doing well on standard chemotherapy and are not candidates for a transplant. Patients with poor risk disease are unlikely to achieve long-term remission with drugs alone and are more likely to be transplanted. Patients with standard risk disease fall into patient-by-patient care (Buchner, 1993).

Growth-factors

Haematopoietic growth-factors are glycoprotein hormones which regulate differentiation and proliferation of progenitor cells and the function of mature blood cells. Recently recombinant haematopoietic growth-factors have been used to reduce the complications associated with chemotherapy and transplantation. For example G-CSF is used to stimulate the production of normal granulocytes and monocytes after transplant which also helps reduce infections. There is an significant increase in the number of CFU-GM in the peripheral blood of patients with Hodgkin's disease in bone-marrow following treatment with GM-CSF (Haas et.al.1992). Peripheral blood stem progenitor cell transplants take about two weeks for blood counts to improve to acceptable levels. It takes longer (up to two years) for full function to recover, especially bone-marrow.

Growth-factor function in haematopoiesis

Bone-marrow cells grown in semi-solid medium eg. agar or methyl-cellulose with purified growth-factors form distinctive colonies. Colonies grown in G-CSF form neutrophils and precursors; those grown in M-CSF form macrophages; those in IL-3 form many different lineages; colonies grown in GM-CSF form neutrophils, macrophages, eosinophils and other cells.

Granulocyte colony-stimulating factor (G-CSF) has a 2.0 kilobase (kb) mRNA transcript which yields an 18-22 kiloDalton (kDa) protein produced by monocyte and fibroblasts. Granulocyte-monocyte colony-stimulating factor (GM-CSF) has a 1.0 mRNA yielding a 14-35kDa protein synthesised by T cells, fibroblasts and endothelial cells. Interleukin 3 (IL-3) is a 14-28kDa protein produced from a 1.0kb mRNA transcript by T cells. Monocyte colony-stimulating factor (M-CSF) has a 4.0 mRNAproducing a 35-45kDa protein produced by monocytes. There is also a 1.8 mRNA M-CSF transcript producing 18-26kDa produced by fibroblasts and endothelial cells. All these CSF are effective at picomolar concentrations *in vitro* and probably less *in vivo*. IL-3 and GM-CSF activities overlap and are both produced by T cells though

in vivo. IL-3 and GM-CSF activities overlap and are both produced by T cells though there is little sequence homology. IL-3 is more active on the earliest progenitors and also able to cause increased platelet, granulocyte and monocyte production. Few normal cells express detectable mRNA for G-CSF, IL-3, GM-CSF except when activated. GM-CSF has different amounts of glycosylation with different biologic activity (heavily glycosylated forms have least activity). GM-CSF acts on earlier progenitors than G-CSF (Clark, 1987).

Clinical administration of G or GM-CSF to patients with myelosuppression causes a rapid recovery and elevation of neutrophil count and primes these cells for bactericidal action and also the production of cytokines by mature neutrophils, monocytes and eosinophils. Two or more factors probably act synergistically in a coordinated fashion to initiate the signal to divide or differentiate. Also one growth-factor may affect the production of its own receptor or that of another growth factor. For example IL-1 has a variety of effects mainly related to inflammation. Stem cell factor (c-kit) acts on pluripotent stem cells and early myeloid and lymphoid progenitors. IL-1 and IL-6 enhance the effects of SCF, IL-3 and GM-CSF on survival and differentiation of early progenitors. EPO, G-CSF, M-CSF, and IL-5 act on later cells which are more committed to one cell lineage, IL-6 also has a role in megakaryocyte formation. Cloned bone-marrow stromal-cell lines constitutively produce only IL-6, M-CSF and require induction by IL-1 to produce GM-CSF and G-CSF. Bacterial infection causes a rapid production of M-CSF, G-CSF, IL-5, 6 and a small rise in GM-CSF within 3-6 hours, there is no detectable IL-3 found however (Nicola, 1989).

Growth-factor effects are mediated through specific receptors on target cells. Many growth-factor receptors belong to a structurally similar receptor family (IL-2 to 6, GM-CSF, G-CSF, Erythropoietin). Ligand binding causes receptor dimerisation initiating a series of phosphorylation events, though there is no direct tyrosine kinase activity. A smaller group of growth-factors (IL-1, M-CSF, SCF) belong to the immunoglobulin super-family which do have intracellular tyrosine kinase activity. Receptor activation may cause changes in second-messenger molecules including cAMP, cGMP, diacyl-glycerol and inositol tri- and tetra-phosphate (IP_3 and IP_4), phosphatidyl-inositol triphosphate and calcium ions. These compounds phosphorylate other proteins involved in signal transduction which may bind together via SH (src homology) domains causing a phosphorylation cascade. The products of RAF (a serine-tyrosine kinase) and MAPK (mitogen activated kinase) transmit signals from cytoplasm to nucleus which activate transcription factors by phosphorylation. Proteins called cyclins are caused to bind to protein kinases in the cdc2 family to form a complex involved in transition of the cell from G1 to S phase and from S into G2 and mitosis (Strauss et.al.1995; Sherr, 1996).

Clinical appplications

Significantly G-CSF given to patients with relapse or refractory leukaemia significantly reduces neutropenia (Byrne et.al.1997). A longer complete remission was found in patients with a high S-phase index presumably growth factors were increasing the proportion of blast cells in cycle. However patients treated with GM-CSF during and after chemotherapy did worse than patients given chemotherapy alone (Buchner, 1993).This may be due to growth-factor-induced blast cell proliferation. Blast cells from different patients obviously may express receptors for combinations different growth-factors and therefore respond to their presence by proliferation or differentiation depending on their relative maturity. Growth-factors also affect the survival and function of normal mature cells with a common mechanism of apoptosis inhibition.

Apoptosis is a genetically-programmed cell suicide pathway which appears to be a default process for cells deprived of positive survival stimuli from growth factors.

Apoptosis

As indicated in the previous section most cells depend on growth-factor support for proliferation and survival. In many cases removal of growth-factors results in the cells entering into a period of quiescence known as G0 (Cory, 1995). If this period is extensive then cells undergo a programmed cell death called apoptosis (Kerr .et.al 1972). Most known chemotherapeutic agents thus far studied also induce apoptosis in target cells, usually through a mechanism which senses the DNA damage caused by these agents. Induction of apoptosis is not limited to highly proliferative cells (Hickman et.al.1994).

Apoptosis is an irreversible cellular process whereby target cells are actively removed from tissues by phagocytic cells following growth factor removal, or detection of cellular damage. It is associated with obvious cellular shrinkage, nuclear condensation and membrane blebbing. In many diseased and normal tissues (eg. during immune function or organ development) cells are actively removed from the surrounding matrix without provoking an inflammatory response

Apoptosis is the loss of cell volume associated with cytoskeletal breakdown resulting in blebbing of the plasma membrane (Wyllie et.al.1984). A variety of active intracellular signalling processes occur, including loss of water, endonuclease activation causing (DNA cleavage at inter-nucleosomal sites (Wyllie, 1980) and fixation of the cell membrane through tissue transglutaminase activation (Piacientini et.al.1991). DNA cleavage however can be partial or even absent when apoptosis occurs (Cohen et.al.1992; Falcieri et.al.1993).

Programmed cell death however and apoptosis may occur through distinct pathways and (PCD) does not necessarily involve inter-nucleosomal DNA fragmentation. Some cells may use both methods, for example TNF-α-induced death of lymphocytes may occur either via apoptotic or non-apoptotic pathways (Cohen, 1993) depending on the culture conditions. Therefore techniques dependent on DNA cleavage eg. agarose gel electrophoresis or *in situ* end-labelling of fragmented DNA, may fail to detect these cells (Sgone et.al.1998; Gorczyka et.al.1993).

Apoptotic cells round up and lose contact with neighbours, there is loss of cell volume (due to secretion of water and ions) causing an increase in density. Membrane blebbing occurs before loss of membrane integrity but probably after nuclear margination and condensation and fragmentation. Nuclear changes in apoptosis can occur without oligonucleosome production (Bellamy et.al.1995).

A number of extracellular agents eg. TNF-α, Fas, chemotherapeutic agents cause activation of sphingomyelinases and release ceramide (from membrane sphingomyelin). Ceramide activates a serine-threonine phosphatase and regulates ICE proteases and the retinoblastoma protein and stress-activated protein kinases. Ceramide levels increase in response to 1, 25-dihydroxy vitamin D3, TNF-α, endotoxin, IFN-γ, IL-1, Fas, CD28, dexamethasone, retinoic acid, progesterone, ionising radiation, chemotherapy heat and nerve growth factor (NGF). Serum withdrawal in leukaemic cells can result in a 15 fold increase in ceramide. The membrane permeable ceramide analogues C2 or C6 may induce apoptosis, senescence, cycle arrest or terminal cell differentiation. Cell cycle arrest induced by ceramide results in dephosphorylation of retinoblastoma protein. Actinomycin D causes ceramide increases by a p53 dependent mechanism. Accumulated ceramide activates CAPP (ceramide activated protein phosphatase) which may result in either retinoblastoma protein activation and cycle arrest or YAMA activation leading to apoptosis (Hanuun. et al 1996).

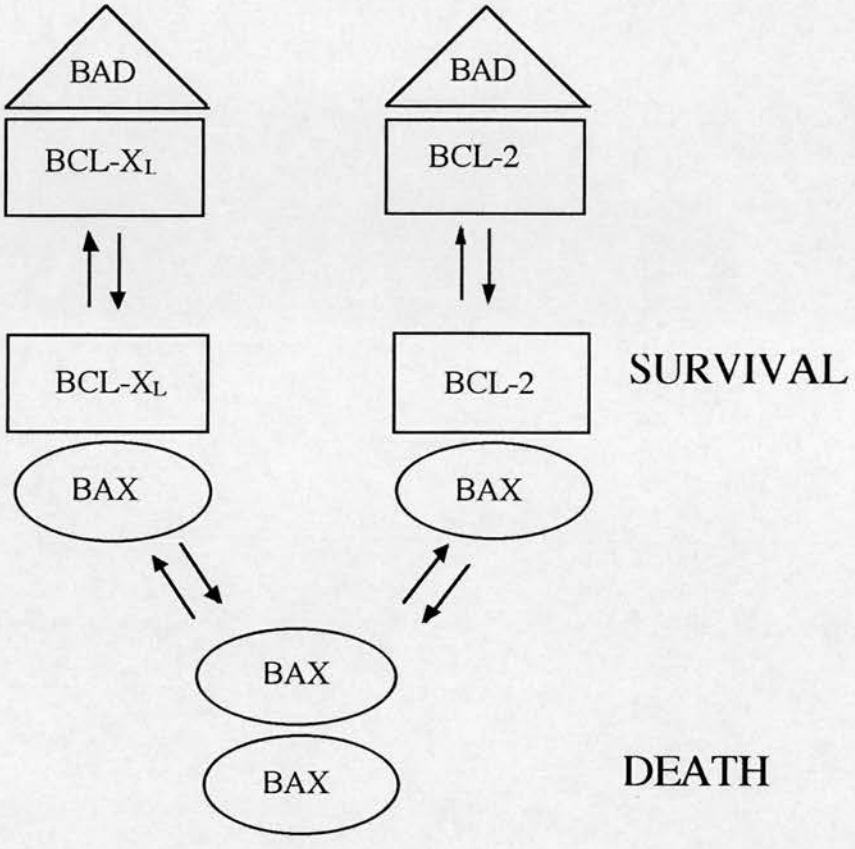
Downstream effects of TNF-α or Fas, Ara C, vincristine, ionising radiation cause an increase in ceramide an effect which can be inhibited by bcl-2 overexpression. In some cell lines however ceramide may protect from apoptosis. Ceramides will induce HL-60 differentiation. CAPP activates protein kinase C through phosphorylation. Ceramide is produced by a sphingomyelinase which may mediate the effects of Ara C, TNF-α or Fas. Ceramide and diacyl glycerol (DAG) do not function solely as signal transduction agents (Hannun, 1996).

Role of bcl-2 in prevention of apoptosis

Myeloid cells have viability factors which suppress apoptosis and are required throughout the differentiation process. The colony stimulating factors (CSF) and IL-3 function as growth-factors, however IL-1 and IL-6 can also function as viability factors without inducing growth, as can PMA or bcl-2. Viability and growth are separately regulated processes. Homeostasis in normal tissue is a balance of cellular proliferation and removal via cell death.

Bcl-2 was characterised from B cell lymphomas with the t(14;18) translocation.where the abnormally regulated protein was shown to cause reduced apoptosis (Zutter et.al.1991; Korsmeyer, 1992). There is a recently characterised family of molecules, structurally related to bcl-2, whose major role appears to be in the maintenance of cellular homeostasis. This bcl-2 family of proteins shares structural homology features known as Bcl-2 homology regions (BH). Cellular death or viability is partly mediated by competitive hetero and homo-dimerisation between pairs of antagonists or agonists. Family members share the bcl-2 homology regions BH-1, 2, 3 or 4 and it appears to

be the ratio of anti-apoptotic to pro-apoptotic members eg. bcl-2 to bax which determines apoptosis (**Figure 3**).



(**Figure 3**)
(Yang et.al.1996)

Hetero- and homodimerisation require the BH-1 and BH-2 regions (Sedlak et.al.1995). For example BH4 regions are conserved among anti-apoptotic bcl-2 homologues. Bcl-2 is located on the inner mitochondrial membrane and nuclear pores

lines. Cells remain in G0 until IL-3 is administered (Hockenbery et.al.1990). In the presence of DNA damage bcl-2 transfected cells were able to survive (Hickman et.al.1994). Similarly 67% of lymphoma cells underwent apoptosis when treated with 5-fluorodeoxyuridine for 24 hours as compared with 8% of cells with a bcl-2 expressing vector (Fisher et.al.1993a). Bcl-2 transfection into cortical thymocytes protects them from radiation, glucocorticoids and anti CD3 antibody induced apoptosis. Overexpression of bcl-2, bcl-xl suppress apoptosis in the absence of growth-factors whereas overexpression of bad, bax, bik override signals from growth-factor receptors and induce apoptosis. Apoptosis suppressor members prevent apoptosis when heterodimerised but are incapable of protecting from apoptosis when heterodimerised with an inducer molecule (Cory, 1995; Kroemer, 1997).

The normal sequence of signalling events may be growth-factor or cytokine binding its receptor causing the activation of phosphoinositide 3-kinase which in turn releases inositol phosphates. These in turn activate Akt (a serine-threonine kinase) and PDK1 which induces phosphorylation of bad-bcl-xl which causes a dissociation of bad from bcl-xl. Bad in turn is sequestered by 14-3-3 (a phosphoserine binding protein) and bcl-xl is able to prevent apoptosis via cytochrome c inhibition (Franke et.al.1997) Levels of bcl-2 or bcl-xl may be regulated by growth-factors which thus maintains cellular viability. Upon growth-factor removal bad is de-phosphorylated, dissociates from 14-3-3 and heterodimerises with bcl-xl. Homo-dimerised bcl-2 or bcl-xl bind to ced 4 homlogues, then bind the inactive form of cysteine proteases. When homodimer binding to ced-4 is disrupted the cysteine proteases become activated ie. normally bcl-2 or bcl-xl homodimers sequester ced-4 and apoptosis is prevented. Heterodimerisation of bcl-xl with bax, bak or bik causes ced-4 release and apoptosis induction. Bad (a pro-apoptotic member) is normally sequestered by 14-3-3 in the cytosol as the phosphorylated (bad) form (Franke et.al.1997).

Not only absolute protein levels (Hu et.al.1996) but post-translational modifications of these proteins may alter functional activity through phosphorylation at serine residues eg. bcl-2 function is lost when phosphorylated (Kroemer, 1997). Disruption of the mitochondrial membrane potential (via apoptotic signals), and therefore permeability transition (PT) pores, causes uncoupling of the respiratory chain, cessation of ATP synthesis, depletion of reduced glutathione (GSH) and nicotinamide-adenine-dinucleotide (phosphate) NAD(P) stores, superoxide production, calcium efflux from the mitochondria and protein leakage. Release of cyochrome c and apoptosis inducing factor (AIF) induce proteolytic activation of caspase (cysteine protease-CPP32) and cause apoptosis. Activation of CPP32 is dependent on the release of cytochrome c from mitochondria (which is normally blocked by bcl-2). Bcl-2 does not affect AIF formation or the effects of cytochrome c (or AIF) on isolated nuclei, again demonstrating the requirement for bcl-2 location on mitochondrial membranes.

Permeability transition inhibitors eg. N-methyl-val-4-cyclosporin A are able to inhibit apoptosis. Bcl-2 and bcl-xl are relatively poor inhibitors of Fas, TNF-α and TRADD (TNF-α receptor associated death domain) death effectors. Inactivation of bcl-2 can be achieved by agents acting on microtubules (eg. taxol and colchicin) through hyperphosphorylation during the G2-M phase of the cell cycle (Kroemer, 1997).

Clinical role of bcl-2 in AML

In patients with AML levels of bcl-2 appear to be correlated to disease prognosis, with patients having high levels of bcl-2 showing a poorer response to chemotherapy (Campos et.al.1993). 82 samples of *de novo* AML were measured for bcl-2 content and it was found that a mean of 23% were bcl-2 positive. The percentage bcl-2 positive cells was higher in M4 and M5 sub-types and those with highest blast cell counts. Bcl-2 levels also were correlated with CD34 expression. High levels of bcl-2 were associated with poor complete remission and shorter survival. The percentage bcl-2 positive cells were highest in monoblastic M4 and M5 and lowest in M3 promyelocytic AML. AML M0-15% positive; M1-25%; M2-14%; M3-8%; M4-35%; M5-29%; M6-35%. KG1-a (an AML cell line) were 97% bcl-2 positive and HL-60 (an APML cell line) 85% positive but with an intensity lower than KG1-a (Delia et.al.1992). In normal bone-marrow bcl-2 was found in mature myeloid cells up to the promyelocyte and erythroblast stages. These authors suggested that high bcl-2 levels in AML was a consequence of the absence of differentiation, rather than deregulated expression (Campos et.al.1993).

Autocrine growth of AML blasts in culture is related to a poor complete remission rate (Lowenberg et al). Autonomously growing AML cells in culture have been shown to have an autocrine GM-CSF-induced up-regulation of bcl-2 (neutralised by anti-GM-CSF antibodies). In patients with non- or partial autonomous growth bcl-2 levels were up regulated by exogenous GM-CSF (Nunez et.al.1994). CD34 positive AML cells were found to express higher levels of bcl-2 than CD34 negative AML blasts. Anti GM-CSF antibodies were able to down-regulate bcl-2, leading to an increased sensitivity to cytotoxic drugs (Bradbury et.al.1997). G-CSF, used to ameliorate chemotherapy-induced myelosuppression in AML, may also enhance proliferation of those tumour cells with the G-CSF receptor. Recombinant human G-CSF is known to enhance the survival of leukaemic AML cells in response to adriamycin and etoposide treatment probably through a bcl-2 mediated mechanism (Kondo et.al.1994).

Some AML cell lines can be induced to differentiate even if they are independent of exogenous growth-factors for growth notably the promyelocytic leukaemia cell line HL-60. Differentiated HL-60 cells have much reduced bcl-2 levels but even before

HL-60. Differentiated HL-60 cells have much reduced bcl-2 levels but even before terminal differentiation these cells re-acquire normal requirements for growth-factors, which if removed causes cells to undergo apoptosis. PMA treatment was able to rescue these cells from apoptosis but was inhibited by amiloride (a Na-H+ antiporter inhibitor) (Sachs et.al.1993). Bcl-2 down regulation in these cells is not sufficient for induction of differentiation. Bcl-2 mRNA down regulation was seen only in the parental cell and not in an adriamycin-resistant form even though both forms were able to differentiate (Blagosklonny et.al.1996).

Antisense

The pivotal role of bcl-2 in the survival of AML blasts, association with poorer survival and in the development of tumour resistance to chemotherapy therefore makes it a central target for drug therapy in AML and other cancers (Campos et.al.1994; Kitada et.al.1994). A novel form of molecular therapy has attracted the attention of many researchers and drug companies (Zamecnik et.al.1978, 1986). This inhibitory therapy uses drugs called called antisense DNA and molecules can be designed with exquisite specificity for known protein targets in disease. By designing a synthetic DNA molecule complementary to the mRNA sequence of a known protein, levels of expression of that protein may be selectively modulated. This has important implications in the design of novel therapeutics since drug development times are reduced with the potential for increased drug specificity and reduced side effects.

Antisense DNA molecules can be targetted to several RNA sites important in the synthesis and assembly of proteins: the AUG site to prevent the translation complex assembly: the 5' cap region to prevent ribosome assembly: the protein coding region to prevent the translation process: or other RNA areas to prevent mRNA transport from the nucleus to cytoplasm (Warzocha et.al.1997). RNAse-H-mediated digestion of RNA has been found to be the predominant mechanism of antisense action in down-regulating mRNA levels (Kirk Field et.al.1995) (**Figure 4**).

(**Figure 4**)

Antisense molecules may prevent the production of proteins by steric hindrance preventing translation, or RNAse H-mediated digestion of the RNA-cDNA heteroduplex.

Many recent reports have suggested good *in vitro* efficacy with selective mRNA targetting and effective down-regulation of target protein function eg. the bcr-abl fusion protein in CML (Chasty et.al.1996), gp120 in HIV (Lisziewicz et.al.1994) using antisense molecules, however large scale clinical trials have been largely unsuccessful with limited effectiveness, and potentially serious side effects. Side effects largely mimic the actions of polyanionic molecules, and as such may affect blood clotting factors (elongation of APTT) (Webb et.al.1997) complement activity and heparin-binding proteins eg. fibrinogen. An antisense phosphorothioate to HIV has been shown to cause mild thrombocytopenia, elevated serum transaminase levels and elongated activated partial thromboplastin times (APTT) with a dose of 2.5mg/kg (Plenat, 1996).

Uptake of Antisense

Cellular studies on uptake of antisense molecules have shown that at 4°C 90% of the antisense associated with cells is localised on the cytoplasmic membrane. However at 37°C the intracellular concentration rises to a plateau after 1-2 hours in serum-free medium. Antisense oligodeoxynucleotides were taken up in a sequence independent manner and were inhibited by plasmid DNA or yeast transfer RNA (Neckers, 1993). This uptake appeared to be a receptor-mediated phenomenon with many authors describing a potential 80kDa cell surface antisense-receptor.

Labelled ligand-receptor complexes internalised by endocytosis display a punctate intracellular fluorescence consistent with patterns of cellular antisense uptake by microscopy (Clarenc et.al.1993). Antisense binding has been described as having a single dissociation constant in the low nanomolar range, with most cells expressing 20-100,000 binding sites per cell. Nuclear, membrane and cytoplasmic staining patterns have also been described. Studies using microinjection of fluorescent-tagged oligodeoxynucleotides have demonstrated a rapid localisation of antisense to nuclear structures which was not dependent on ATP or temperature (Leonetti et.al.1991)

Recent reports have suggested that CD11b is a potential mechanism by which antisense phosphorothioates can be internalised into cells (Benimetskaya et al). Soluble fibrinogen, but not an GRGDSP peptide, was able to inhibit binding of a 15mer antisense to HL-60 cells by up to 40%. Synthetic heparin analogues were also able to inhibit antisense binding to both the αM and β2 subunits of Mac-1. Both αM and β2 subunits are believed to contain heparin binding sites and a metal ion-dependent adhesion site (MIDAS) which bind Ca^{2+} and Mg^{2+}. Phosphodiester antisense Mac-1 binding was shown to be Ca^{2+} or Mg^{2+} dependent whereas phosphorothioate (where

an acidic oxygen is replaced with sulphur) was not.

Mac-1 is a member of the leucocyte integrin family and has been shown to bind ICAM-1, fibrinogen, C3bi, factor X, heparin. The β2 integrins share the non-covalently associated β chain CD18 and differ only in their alpha sub-unit: LFA-1 = CD11a-CD18: Mac-1 = CD11b-CD18: p150,95 = CD11c-CD18. Alpha subunits share a conserved 200 amino acid I (inserted) domain which contains a heparin-binding site and three tandem divalent-cation binding repeats (MIDAS or EF-hand domains). Antibodies to the EF region however had no effect on phosphorothioate binding.

Because of its pivotal role in the maintenance of cellular homeostasis and elevated levels in poor prognosis AML makes bcl-2 bt an ideal target for antisense therapy. The effectiveness of 4μg/ml of cytosine arabinoside has been shown to be more effective in the presence of antisense to bcl-2. The highest intracellular antisense phosphorothioate content was achieved at 4-5 days which accounts for the delay in its effectiveness (Campos et.al.1994). A 16mer antisense phosphorothioate to IL-1β converting enzyme (ICE) at 10-75μM inhibited proliferation of peripheral blood and bone-marrow leukaemic cells and inhibited CFU-L formation. IL-1 has been shown to be important in initiating paracrine and autocrine growth of AML blasts (Stosic-Grujicic et.al.1995). Antisense bcl-2 has also been shown to down-regulate lymphoma growth in SCID mouse model (Cotter et.al.1994) and in human lymphoma (Webb et.al.1997). Wilms tumour gene product (WT) may also be a therapeutic target for antisense therapy in AML since it too is associated with a poor prognosis (Olsson et.al.1996) and was found to be expressed in all of 96 patients with AML or CML. There were no effects of WT antisense on normal CFU-GM but leukaemic colonies were inhibited. A WT cDNA driven by a CMV promoter abrogated antisense efects as determined by flow cytometry (Yamagami et.al.1996).

U937 cells (a leukaemic cell line with monocyte-macrophage characterisics) treated with c-myc antisense by electroporation caused rapid cell death (presumably through an antisense effect). The treatment appeared to have little effect on murine hematopoietic cells. Electroporation causes transient membrane pore formation, and with optimisation 100% transfection and suppression of c-myc and decreased cell viability can be achieved (Bergan). U937 cells have low levels of bcl-2 but when transfected with a vector containing bcl-2 they increased their resistance to cytosine arabinoside by 3 logs (Suresh et.al.1996).

Aims & Objectives

This thesis has set out to detemine bcl-2 levels, monitor the delivery dynamics and effectiveness of antisense uptake in AML and normal blood cells in an attempt to obtaining either an effective purging protocol which could be used to remove tumour cells from bone-marrow and apheresis products, or to provide a more effective therapy for the treatment of refractive and resistant AML.

Growth patterns of AML blast growth *in vitro* have been shown by others to be related to bcl-2 protein levels and subsequent disease prognosis. These parameters were therefore characterised. Values for bcl-2 protein expression in normal versus AML cells were obtained.

In an attempt to reduce the leukaemic cells' resistance to chemotherapy (mediated through bcl-2) an antisense directed to bcl-2 protein was used to either effect leukaemic cell cytotoxicity directly or to increase their sensitivity to standard chemotherapy. Antisense uptake into normal and leukaemic cells was characterised and the mechanisms of internalisation sought with a view to improving the selectivity of drug action towards tumour cells. The functional effectiveness of antisense was monitored by development of a sensitive assay for apoptosis using Annexin V staining of cells.

Materials and Methods

Flow Cytometry Reagents

Phosphate buffered saline (PBS)	(1 litre)
NaCl (0.9%)	9.0g
KH_2PO_4	0.144g
Na_2HPO_4	0.421g

Permeabilisation buffer (for bcl-2 measurement)	
70% Methanol in PBS	(1litre)

PBS-Azide-BSA (PAB) buffer	(1 litre)
1% Bovine serum albumin (BSA)	10g
0.1% NaN_3 (Sodium azide)	1g
PBS	to 1 litre

FACS Flow™ Becton Dickinson (Sheath fluid)	(20 litres)

Cell preparation for flow cytometry (FACS) analysis:

Cells were harvested and adjusted to 10^6 cells/ml, washed twice (200g, 5 minutes) and resuspend in 1ml PBS. An appropriate amount of titrated labelled antibody was added and incubated for 20 minutes on ice in the dark (to avoid photo-bleaching and to reduce non-specific reactions). Cells were then washed twice with PBS as before. If required a permeabilisation step for cytoplasmic antigen (bcl-2) detection was carried out after staining for surface antigens as above (Aiello et.al.1992). Cells were resuspend in 1ml of 70% methanol-PBS and incubated for 20 minutes at 4°C (Sartor et.al.1994; Jung et.al.1997). Cells were then washed twice and resuspend in 1ml PBS. Where required, non-specific antibody binding to Fc_γ receptors was blocked by incubating the cells with 10µl non-immune, non-labelled isotype control for 20 minutes at 4°C. Cells were then washed with PBS and stained with the labelled antibody for 20 minutes at 4°C, then washed as usual in PBS. Finally the cells were resuspend in 300µl PBS for immediate FACS analysis (or in PAB if there was any delay prior to staining).

Flow cytometry (FACS) analysis

To ensure reliable performance and repeatibality between data FACS calibration was performed on a regular basis usiing Calibrite™ beads (Becton Dickinson #95-0002) according to the manufacturers protocol.

CellQuest™ software (Beckton Dickinson, Oxford UK) was loaded and connected to the cytometer; appropriate instrument settings for each cell type set; the required number of events (eg.routinely 20-30,000 cell "events" or 50,000 for rare cells such as CD34+ cells); acquisition plot set on forward-scatter (size) versus side-scatter (granularity); parameter description set to the fluorescent antibody label then data acquired.

Data analysis

A bivariate analysis plot of forward versus side scatter was initially used to determine the size and granularity of the cells under study. A plot of the required parameters was then prepared, for example CD34 expression versus side scatter gave the best visualisation of stem cells (low granularity and high CD34 expression). It was then necessary to gate the population of interest and subsequently plot a third, or fourth parameter for example bcl-2 levels on a histogram plot of CD34 positive cells (CD34 versus SSC). Histograms were compared by Kolmogorov-Smirnov statistics where a value of $p<0.05$ was considered significant.

CD34 positive cell determination in AML and normal peripheral blood and bone marrow

A mononuclear cell preparation (10^6 cells/ml) of blood or bone-marrow was stained with 10μl CD34-PE monoclonal antibody (HPCA-2, Beckton-Dickinson, Oxford, UK). CD 34+ cells were identified on the basis of low side scatter and CD34 positivity. This is a simplified version of the recently adopted ISHAGE protocol, namely : firstly a histogram of CD45+ve (gate R1).was identified, secondly a histogram of CD34+ve (gate R2) cells was identified. A CD45 versus side scatter dot plot (identified blasts with low side scatter low CD45 (gate R3) and finally a FSC versus SSC plot confirmed these cells were contained within the lymphoid and blast region (Sutherland et.al.1994).

Quantitation of antibody binding

The Quantum Simply Cellular™ (Sigma, UK) kit was used for antibody quantitation. It contained a mixture of microbeads coated with five differing capacities for antibody binding. The fluorescence signal from the peak channel was used to construct a calibration curve from which unknown values were determined (**Figure 9b**). Cells under study and 50µl of microbeads (approximately 10^5 beads) were dispensed into a test tube and labelled with enough test antibody to ensure saturation. Beads and cells were prepared in the same manner and processed for flow cytometry as indicated above and the flow-cytometer aligned for optimal detection of cells and beads. The microbeads were analysed by gating on the singlet population and the peak fluorescent channel determined for each bead population. A regression line was plotted between the standard points of antibody-binding capacity (ABC) versus peak fluorescence channel (linear scale-channel numbers) and unknown values extrapolated from the equation of the regression line (**Figure 9b**).

Stable cell labelling

PKH2-GFL stable green fluorescent cell linker was used to monitor the sensitivity of flow cytometric detection of tumour cells populations in culture in the MRD experiments. PKH2 is an aliphatic fluorescent chromophore for labeling membrane lipid. It has an excitation wavelength of 488nm and emission wavelength of 504nm, propidium iodide has an emission wavelength of 570nm (Horan et.al.1990). Fluorescent red blood cells intensity remained relatively constant over 60 days using 10^{-5}M PKH-3 (Slezak et.al.1989; Traycoff et.al.1995).

Firstly a single cell suspension (2 x 10^7 cells per stain) was prepared and cells washed once in IMDM (without serum) at 200g for 5 minutes at 25°C. The supernatant was aspirated and cells resuspend in diluent A. The dye was prepared in diluent A at two times final concentration in a polypropylene tube. Cells were then added to the dye and mixed immediately and incubated for 5 minutes at room temperature. The reaction was quenched with 2ml foetal calf serum (FCS) and incubated for 1 minute. Two volumes of complete medium were added and cells washed three times, cells resuspend in an appropriate volume of medium and the analysis completed. Varying cell and dye concentrations were required to optimise staining conditions for each cell population.

Cell culture reagents

Red blood cell lysis

20 ml samples of anti-coagulated blood (100µl preservative-free heparin) were lysed in a hypotonic solution using 4-10 volumes of ammonium chloride red cell lysis buffer. Leucocytes were sedimented at 200g and the haemolysed supernatant aspirated. The lysis and centrifugation step was repeated if neccessary until a clear solution was obtained. The supernatant was again aspirated and discarded and the resulting cell pellet resuspend in 1ml of medium and cell viability counted using trypan blue.

Red blood cell (RBC) lysis buffer	(1 litre)
Ammonium chloride (NH_4Cl):	8.26g
Potassium bicarbonate (KH_2CO_3):	1.00g
Na_2 EDTA:	0.0037g

This solution was filter-sterilised with a 0.2µm filter and renewed within one month.

Tissue culture medium (500ml)

10% foetal-calf serum (FCS heat-inactivated for 30mins at 60°C)	50ml
1% Penicillin (50U/ml)-Streptomycin (50µg/ml) solution	5ml
1% L-Glutamine solution	5ml
IMDM (Iscove's Minimal Dulbecco's Medium)	500ml
Trypsin-EDTA for harvesting adherent cell lines	0.25%
Hanks Balanced Salts Solution (HBSS)	
Trypan blue (0.2%) cellular viablity stain:	

Cellular viability methods and reagents

0.2% Trypan blue in PBS was used for routine determination of cellular viability; viable cells exclude the stain, whereas dead (or permeabilised) can not, therefore stain blue. Briefly 10µl cells were mixed with 90µl trypan blue and 10µl of the mixture counted immediately. The cell count per ml was given as the number of viable cells in one large hemacytometer square x 10^5.

Propidium iodide (PI) solution (0.25%) was used for determination of cellular viability by flow cytometry. Viable cells exclude the stain, whereas dead (or permeabilised) cells take up the stain into their nuclei whereupon the quantum yield of the propidium iodide (PI) fluorescence increases and the cells emit a bright fluorescence. A 0.025% stock solution used at 40μl per ml of cells.

Fluorescein-diacetate (FDA) solution was also used for determination of cellular viability. Viable cells contain non-specific esterases which cleave fluorescein-diacetate into free fluorescein (being highly charged, cannot diffuse across intact membranes) therefore intact cells fluoresce brightly with a green fluorescence, dead (or permeabilised) cells however, allow the stain to diffuse out of the cell and do not fluoresce. A stock solution of 5mg/ml was made in acetone, aliquoted and stored at -20°C in the dark. A working solution of 10μl stock solution was dissolved in 1ml of PBS. 5μl of this was then added to 50μl cell of suspension (10^6 cells/ml), mixed and left for 15 minutes at room temperature. The cell sample was centrifuged at 200g, the supernatant discarded and cell pellet resuspended in PBS and analysed for viability.

Mononuclear cell (MNC) preparation

Freshly harvested, anti-coagulated (1000U/ml PumpHep preservative-free heparin, Leo Laboratories, Bucks, UK-100μl per 20ml) blood or bone-marrow samples were processed using aseptic conditions (laminar-flow cabinet). An equal volume of sterile Hanks Balanced Salt Solution (HBSS Sigma, UK) was added and the diluted sample layered carefully onto Lymphoprep™ solution in a conical tissue-culture centrifuge tube. The sample was then centrifuged at 700g for 15 minutes to pellet the erythrocytes. Mononuclear cells (comprising monocytes and lymphocytes) were carefully removed from the interface and washed twice with 20ml HBSS by centrifuging at 200g for 5 minutes at 4°C. The mononuclear cell pellet was resuspended in 1ml HBSS and cellular viability assessed using trypan blue as described above prior to further processing.

Culture of cell lines

Cryopreserved cell pellets were rapidly thawed in the 37°C water bath. The cells were then tranferred to 1ml thawing mixture and incubated for 1 minute. 10mls of IMDM was then added and the mixture incubated a futher 10 minutes. Cells were then washed twice and resuspended in IMDM, and cellular viability assesed using trypan blue.

KG1-a cells (Koeffler et.al.1978) were cultured as a suspension in IMDM (containing 20% FCS, 2mM L-glutamine, and penicillin (0.5U/ml) + streptomycin solution (0.5μg/ml) and maintained at a density of between 10^5-10^6 cells per ml. Medium was renewed every 3-4 days and the cells sub-cultured following removal of dead cells by layering over Lymphoprep™ and collecting viable cells at the interface. Cells were then plated out in fresh medium at 10^5 cells per ml and cultured at 37°C, 5% CO_2.

Immunophenotyping of KG1-a cells was performed using appropriate acute leukaemia monoclonal antibody panels by Mrs Anita Ford (Haematology Department Western General Hospital, Edinburgh). Cells were found to be positive for CD34 (98%), HLA-DR (1%), CD7 (93%), CD15 (74%), CD33 (1%), CD13 (98%), CD45 (100%) and negative for CD19, 10, 20, 5, 4 and 3.

HL-60 and other cell lines were grown in IMDM with 10% FCS and the medium renewed every 3-4 days.

Blast cell colony (BCCA) assays

5637-conditioned medium (5637-CM) was used as a source of colony stimulating factors for the blast cell colony assay (BCCA) (Gabrilove et al). A cryopreserved cell pellet (10^7 cells) was rapidly thawed from liquid nitrogen storage in a 37°C water bath. The thawing mixture comprised 20ml FCS, 2ml heparin (1000 IU/ml), 2ml DNAse (20,000 IU/ml), 2ml $MgSO_4$ (0.15M). Thawed cells were added to 20ml of HBSS and washed twice at 200g for 5 minutes, 4°C. The cell pellet was resuspended in 10ml of pre-warmed complete medium (IMDM with 10% FCS) then added to a $25cm^2$ tissue-culture flask and transferred to a 37°C humidified CO_2 incubator (5%) until cells were confluent, replacing the medium every 3-4 days. When the cells were confluent, the medium was discarded and the cells washed twice with HBSS. For further sub-culture cells were trypsinised with 2ml of 0.25% trypsin-EDTA for 10 minutes at 37°C to remove adherent cells. The trypsin was inactivated with 5ml FCS. The cells were collected and washed in HBSS, resuspended in complete medium and split into two or more culture flasks for further culture. For preparation of conditioned medium (CM) fresh medium was applied to confluent cells and left for 5-7 days to allow growth-factor production. The medium was then aspirated, centrifuged at 200g to pellet debris, the supernatant was then filter-sterilised through a 0.2μm filter and batches pooled as a source of growth factors (IL-1, IL-3, GM-CSF (Gabrilove et.al.1986) for use in the blast cell colony assay.

Methyl-cellulose preparation for blast cell colony (BCCA) assays

3.2g Methyl-cellulose (Sigma) were autoclaved in a 200ml glass bottle, 100ml distilled water with a magnetic stirrer were autoclaved in a separate bottle. Powdered IMDM culture medium (Gibco, Paisley UK) was added to 500ml distilled H_2O to which had been added 3.0g $NaHCO_3$ to give a 2 x IMDM stock solution which was sterilised using a 0.2μm filter. The sterile methyl-cellulose was added to the sterile hot water, mixing regularly. When the solution was completely mixed it was transferred to the microwave for 10 minutes at the lowest power setting and allowed to simmer. When cooled to hand temperature, 100ml of pre-warmed 2 x IMDM stock was added to give a final mixture of 1.6% methyl-cellulose in IMDM. The mixture was then transferred to the cold room (4°C), with constant stirring to allow the mixture to gel. When cool, the mixture was aliquoted, centrifuged at 2000g for 10 minutes to remove debris and stored at -20°C until required.

For blast cell colony assay patient samples (peripheral blood or bone marrow) were T cell depleted with an excess of anti-CD2 Dynabeads (Dynal, Norway #111.01) to remove a potential source of growth factors. Samples were grown in the presence or absence of 5637-conditioned medium (5637-CM), and in the presence or absence of serum to look for leukaemic blast colony formation. 2.5 x 10^6 mononuclear cells were isolated from blood or bone-marrow as described above. Briefly anti-CD2 Dynabeads were added at an optimal bead-to-target ratio and incubated at 4°C on a tilting and rotating shaker for 30 minutes. After the incubation a 5-fold excess of IMDM was added and the tube placed in the magnetic particle concentrator (Dynal, MP-1) for 2-3 minutes to collect the rosetted cells and unbound beads. Depleted supernatant was transferred to a second tube, centrifuged at 200g for 5 minutes, and the cells resuspended in 300μl IMDM at a concentration of 2 x 10^6 cells/ml.

450μl aliquots of the following were prepared in a sterile tube:

A) For serum-containing cultures.

10,000μl of 1.6% methyl cellulose in IMDM; 2000μl of foetal calf serum; 200μl penicillin (50U/ml stock) + streptomycin solution (50μg/ml stock) solution; 200μl L-Glutamine (2mM stock solution): 2000μl of growth factor (if required, otherwise 2000μl IMDM); 3600μl IMDM. 50μl cells (2 x 10^6 cells/ml) were added to each 450μl mixture.

B) For serum-free cultures

10,000µl of 1.6% methyl cellulose in IMDM; 200µl penicillin (50U/ml stock) + streptomycin solution (50µg/ml stock) solution; 200µl L-Glutamine (2mM stock solution): 2000µl of growth factor (if required, otherwise 2000µl QBSF-51); 5600µl IMDM. 50µl cells at 2 x 10^6 cells/ml was added to each 450µl mixture.

Suspensions were prepared in sterile tubes, mixed thoroughly and allowed to stand for 5 minutes to allow bubbles to rise. A row of irrigation wells (100µl HBSS) was placed around the wells of a 96-well flat-bottomed tissue-culture plate to prevent colonies drying out. The cell suspensions were then plated out in triplicate on 96-well plates in 100µl aliquots (20,000 cells per well) and incubated for 7 days. The number of colonies containing more than 20 cells (approximately) was counted at three, five and seven days after initiation of culture. The Autostimulatory Index (ASI) was determined as folows

$$\frac{\text{number of colonies per } 2\text{x}10^4 \text{ cells in NCM}}{\text{number of colonies per } 2\text{x}10^4 \text{ cells in 5637-CM}}$$

Group 1 fail to grow at all have an ASI of zero.
Group 2 grow only in the presence of growth factors have an ASI of less than 0.1.
Group 3 show autonomous growth but are further stimulated by growth factors have an ASI of between 0.1 and 0.8.
Group 4 show autonomous growth which cannot be further stimulated and have an ASI greater than 0.8 (Reilly et.al.1989a)

MACS™ immuno-magnetic enrichment of CD34+ cells

This method was essentially adopted from the the CD34 positive isolation kit protocol (Miltenyi code # 467-01). Briefly mononuclear cells were prepared as described above from bone marrow or peripheral-blood stem-cell harvests using an anti-coagulated sample (preservative-free heparin., Leo Laboratories, Bucks, UK). The washed cell pellet was re-suspended in 300µl HBSS per 10^8 cells and 100µl of reagent A1 (IgG_1) and 100µl of Reagent A2 (anti-CD34 QBEND-10-biotin) added per 10^8 cells, mixed gently and incubated for 15 minutes at 4°C. The cells were then washed in 5ml HBSS, the supernatant completetly removed and cells resuspend in 400µl HBSS. 100µl of reagent B (magnetised Streptavidin) was added, the sample mixed gently and incubated for 15 minutes at 4°C. Cells were washed in HBSS and resuspend in 400µl of sterile, de-gassed PAB. The cells were then added to a pre-filled mini-MACS™ column (type

MS without flow-restrictor) and CD34 negative cells allowed to flow through and the column washed with 4 volumes of PAB. The column was then removed from the magnet, 1ml PAB added and CD34 positive cells eluted using the plunger; this step was repeated, if required to increase recovery, with a fresh column and 500μl PAB for further enrichment. To monitor the enrichment process a sample of the cells was stained with with anti-CD34 (HPCA-2 PE) and analysed by flow cytometry to assess recovery (Miltenyi et.al.1994).

Antisense oligo-deoxynucleotides

Most antisense oligo-deoxynucleotides (oligos) were used at a working concentration of 5-15μM as the phosphorothioate derivative since this is more resistant to nuclease digestion. Labelled oligos were labelled *in situ* during synthesis at the 5' end with FAM (Fluorescein Addition Monomer). Upon receipt oligos were dissolved in distilled water, filter sterilised with a 0.2μm filter, aliquoted and stored at -20°C until required.

Bcl-2 antisense sequence (**5** ' TCTCCCAGCGTGCGCCAT); scattered (nonsense) sequence (**5** ' TGCACTCACGCTCGGCCT) were obtained from Oswell DNA, Southampton, UK).
Control antisense phosphorothioate (**5'** CTCTCGCACCCATCTCTCTCCTTCT) in Biotinylated and FAM-labelled forms were obtained from Hybridon Ltd, Cambridge, Mass, USA).

Streptolysin O reversible cell permeabilisation

Streptolysin O (Sigma, UK) was used for the reversible permeabilisation of viable cell membranes to allow uptake of macromolecules, specifically antisense oligo-deoxynucleotides (Spiller et.al.1995). A range of enzyme concentrations and incubation times were studied to monitor the most appopriate conditions for reversible permeabilisation. Streptolysin O permeabilisation buffer was 137mM NaCl, 100mM PIPES, 5.6mM Glucose, 2.7mM KCl, 2.7mM EGTA, 1.0mM NaATP, 0.1% BSA. Briefly cells for permeabilisation were isolated, washed and resuspend in premeabilisation buffer. An appropriate concentration of antisense was added to the mixture and cells incubated in the presence of an optimal concentration of Streptolysin O for 5-15 minutes at 37°C. After incubation cells were washed in complete medium and membranes allowed to reseal for 2 hours. Cells were then washed again and harvested, ready for subsequent analysis.

Liposomal transfection

A solution of antisense was made in serum-free medium (QBSF-51). A solution of Lipofectin™ was also made up in serum-free medium (QBSF-51) and incubated for 10 minutes at room temperature. The two solutions were mixed together for 15 minutes at 37°C prior to addition to cells to allow micelle formation. Cells were incubated in the presence of the liposomal-antisense mixture for 4 hours at 37°C, 5% CO_2. Cells were then washed twice in serum-free medium, surface stained with fluorescent antibody when required, and analysed.

Apoptosis detection

Annexin V was used as a marker for early-stage apoptosis. Prior to nuclear condensation and fragmentation cells undergo changes in membrane structure, exposing internal phosphatidylserine residues on the surface (Fadok et.al.1992). This was detected by the calcium-dependent molecule Annexin V which avidly binds phosphatidylserine residues. Annexin V was obtained as the FITC conjugate from Biowhittaker Inc (Bender Med Systems, UK). Annexin buffer was made as follows; 10mM HEPES, 150mM NaCl, 5.0mM KCl, 1.8mM $CaCl_2$, 1.0mM $MgCl_2$. Cells were harvested, washed twice and resuspended in annexin buffer. 10μl Annexin V-FITC (2.5μg) was added per 10^6 cells and 20μl propidium iodide stock added immediately prior to assay in order to determine viability. Live healthy cells were Annexin-V negative, PI negative; dead (necrotic and late apoptotic) cells were Annexin-V positive, PI positive; early apoptotic cells were Annexin-V positive, PI negative.

A range of reagents with differing mechanisms of action were chosen to induce apoptosis. Di-butyryl cyclic-adenosine mono-phosphate (cAMP 1mM). Cycloheximide (CHX 50μM) This molecule prevents *de novo* synthesis of protein molecules by interfering with the message translation step. Actinomycin D (Act D 4μM). This drug binds double stranded nuclear DNA, intercalates into the groove and prevents DNA replication and *de novo* mRNA synthesis. Cytosine Arabinoside (AraC 1μM. This drug is incorporated into nuclear DNA in place of deoxyribocytosine and prevents elongation and causes strand breaks. All of the above stock solutions were made up in IMDM and filter-sterilised with a 0.2μm filter.

Annexin + Antibody staining

Fluorescent surface antibody staining was carried out as indicated above. 10^6 cells were then washed twice and resuspended in 1ml in annexin buffer. 10μl Annexin V-FITC was added and incubated for 10 minutes in the dark at 4°C. Cells were then washed twice and resuspended in annexin buffer prior to analysis.

Annexin + PI (pemeabilised)

Annexin surface staining was performed as described. Cells were then resupended in 1ml of 70% methanol in annexin buffer and permeabilised for 20 minutes at 4°C. Cells were then washed twice in annexin buffer and 30μl propidium iodide solution added and incubated for 60 minutes at 4°C. Cells were analysed by FACS without washing.

Antibody + DNA staining

Cells were stained for surface or cytoplasmic markers as described above. Cells were then resupended in 1ml of 70% methanol in PBS and permeabilised for 20 minutes at 4°C. Cells were then washed twice in PBS and 30μl propidium iodide solution added and incubated for 60 minutes at 4°C. Cells were analysed without washing.

Cell morphology

Morphology was assesed on May-Grunwald Giemsa stained cytospins of 10^5 cells using the automated staining equipment in the Haematology department of the Royal Infirmary, Edinburgh

MTT dye cell proliferation assay

The tetrazolium salt MTT (Sigma #M-5655) was used as an index of cell proliferation on supension cultures. Briefly a single cell suspension was added to a 96 well culture plate. Cells were plated out at differing densities in order to determine the most suitable for analysis. Once selected, this density of cells was used for the remainder of the experiment. The agent of interest was added to cells at the optimal density and 10μl of MTT stock added for the final 4 hours (minimum) in order for dye conversion (to an insoluble blue formazan dye) to take place. After incubation the cells were harvested by adding 100μl of a pre-warmed iso-propanol-HCl (0.3M) mixture to lyse the cells and solubilise the dye. Optical density was measured at 570nm and the result extrapolated from a standard curve (generated with known cell densities). The tetrazolium salt (MTT) is reduced to an insoluble formazan, the optical density of which is related to cell density extrapolated from a standard curve.

Fluorescence microscopy

Following staining with fluorescent markers, and analysis by flow cytometry, cells were prepared for microscopy. 50μl cells were added to a well on a microscope slide and analysed under water-immersion lenses at x400-1000 magnification. For preparation of 35mm slides 30 second exposures for fluorescent cells and 10 second exposures for transmitted light were taken on Ektachrome 400ASA slide film.

Confocal microscopy

Samples were incubated with antisense as described above, surface stained with fluorescent antibody and 0.1μm optical sections analysed under ultraviolet (UV) illumination with a confocal microscope (Zeiss). Digital images (PICS) were saved to disk or printed.

Fluorescence quenching agents

In order to deteremine membrane versus cytoplasmic fluorescence a variety of quenching agents were tested.
0.2% Trypan blue solution 0.2g/100ml (Sigma 34078) (van Amersfoot et.al.1994).
0.2% Crystal violet solution 0.2g/100ml (Sigma 34024) (van Amersfoot et.al.1994).
1% Tannic Acid solution 1g/100ml (Sigma T-0125) (Giaimis et.al.1994).

Fluorimetry was performed on a Perkin-Elmer fluorimeter. With reference to a PBS blank the fluorescence of solutions of antisense was determined in the presence and absence of the quenching agents indicated above.

Polyacrylamide gel electrophoresis (PAGE)

Native and denaturing polyacrylamide gel electrophoesis (PAGE) was used to reveal the presence of potential secondary structure formation in the antisense molecules.
18% polyacrylamide gels were prepared as follows:
0.3ml distilled water; 4.5ml acrylamide stock; 4.5ml bis-acrylamide stock; 0.5ml Tris Acetate EDTA (TAE buffer) (x20); 100μl Ammonium persulphate; 10μl (TEMED).
For denaturing gels 7M uea (4.2g) was also included in the mixture.

Gels were run at 100Volts, 20milli-Amps until the dye front was three-quarters down the gel. DNA bands were revealed with addition of 25μl ethidium bromide solution to the gel following removal from the apparatus. The gel was de-stained in distilled water prior to photography.

Antisense conjugation to poly-carboxylate beads

A 5'-NH_2-linked oligodeoxynucleotide in the sense orientation was conjugated to polcarboxylate microspheres using the EDC reagent (Peirce, UK). 5'-labelled complementary antisense was added in excess and allowed to hybridise to the linked sense DNA on the beads. Versions of the complementary sequence were allowed to hybridise independently a) 5'-FAM-labelled antisense; b) 5'-Biotinylated antisense. Molar equivalences for each of the antisense were thus determined by hybridising to a fixed quantity of beads and measuring 1) green fluorescence (FL-1) and 2) red fluorescence (FL-2) signals.

Results

Annexin assay development for apoptosis quantitation

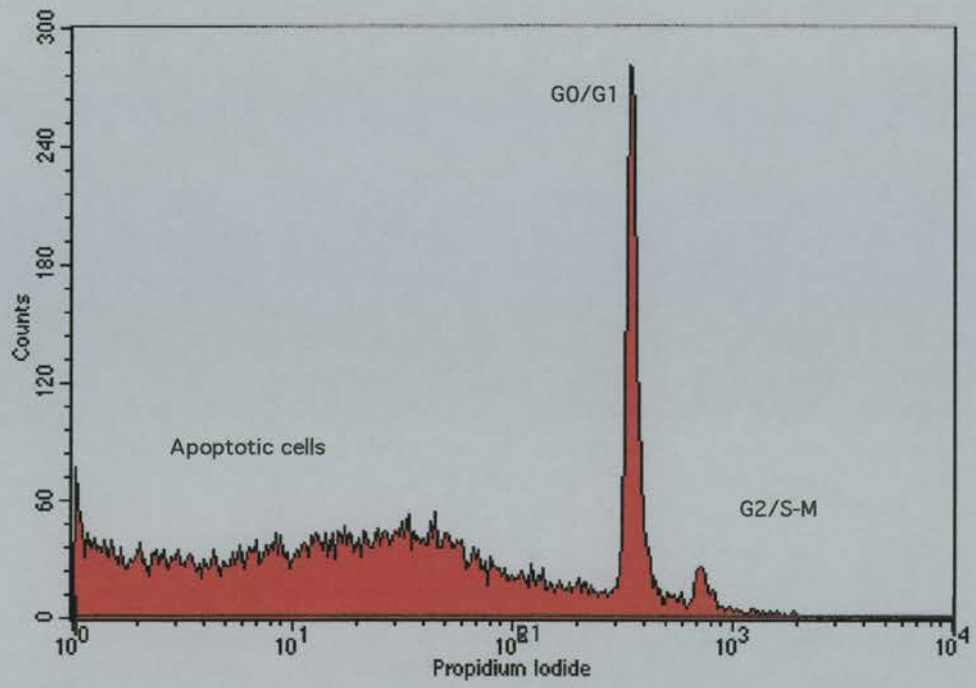
Defects in apoptosis signalling mechanisms are believed to be an important factor in the development of cancer (Ashwell et.al.1994). Apoptosis has been found to be an important mechanism in the action of many chemotherapeutic drugs (Kaufmann, 1996) and in the depletion of CD4 positive cells in HIV (Laurent-Crawford et.al.1998). Therefore the use of a simple, rapid and reliable method of evaluating the rate of apoptosis may allow clinicians to monitor anti-cancer treatment in a dynamic fashion and help assess novel therapeutic strategies. *In vitro* detection and quantification of apoptosis may therefore be useful in the prediction of the sensitivity of tumours to chemotherapy prior to initiation of treatment.

Many different types of anticancer drugs with disparate intracellular targets have been shown to induce apoptosis in susceptible cells (Hickman et.al.1994). There was therefore a requirement for a rapid method of identification and quanititation of the degree of apoptotis in cell populations. Obvious histologic findings include shrinkage, nuclear condensation and fragmentation, with membrane blebbing, however this gives limited information regarding cellular phenotype. Similarly it is often difficult to quantify apoptotic cells within dynamic tissues, since these cells are rapidly removed by phagocytes although apoptotic cells may remain recognisable within tissue for 4-9 hours (Arends et.al.1991). There was therefore a need for development of an assay which could be readily quantified which also allowed detection of early stages of apoptosis before cells were removed from tissues.

Several established flow cytometric methods have been used to quantify populations of apoptotic cells. Most have hitherto depended on changes in staining patterns seen using DNA-binding dyes. These depend on disturbances in membrane permeability or on the presence of modified chromatin found in necrotic or apoptotic cells altering the spectral characteristics of these dyes (Nicoletti et.al.1991).

Quantitative and qualitative changes in apoptotic cells have been assesed by measuring the DNA hypodiploidy of cells (**Figure 5**). This labelling may be carried out on whole cells, but more commonly utilises propidium iodide (PI) staining on isolated nucleii (Telford et.al.1992) to measure the DNA fluorescence histograms of lysed cells (Nicoletti et.al.1991). This hypodiploid fluorescence histogram of DNA content is easy to discriminate from normal cellular DNA content, however this technique depends wholly on DNA fragmentation which is not always present in apoptosis or programmed cell death. This hypodiploid (sub-G1 or A0 peak) is due to an increased

amount of low molecular weight DNA within these cells.



(**Figure 5**). Flow cytometric histogram of propidium iodide stained cells revealing the different stages of the cell cycle and apoptotic cell (sub-G0) regions.

This method also allows determination of cells populations within different phases of the cell cycle (Dive et.al.1998) and measurement of numbers of apoptotic, dead and live cells. Apoptosis can be seen to be selective to cells within particular phases of the cell cycle eg. S phase in myelogenous leukaemias (Del Bino et.al.1991) and G0 phase in thymocytes (Bruno et.al.1992).

Membrane permeability to DNA binding dyes is often combined with chromatin changes and differential binding of DNA intercalating dyes to measure apoptosis. Low permeability dyes such as propidium iodide, or 7-amino-actinomycin D (7-AAD) discriminate cells with intact membranes and are normally combined with a second dye

eg. Hoechst 33342 which enters all cells and differentially stains the DNA of cells with intact nuclei (viable) and of cells with an altered chromatin conformation (apoptotic and necrotic cells (Dive et.al.1998; Ormerod et.al.1992; Ormerod et.al.1993; Schmid et.al.1994). Cell membrane permeability however, only gauges the integrity of nuclear and plasma membranes to DNA binding dyes such as ethidium bromide (EtBr) (Lyons et.al.1992), propidium iodide (PI) (Mower et.al.1994), 7-amino-actinomycin D (Schmid et.al.1992).

Apoptotic cells have been shown to have a high degree of DNA breaks or nicks (Meyaard et.al.1992) which can be detected by the TUNEL (TdT-mediated dUTP nick-end labelling) technique (Sgone et.al.1998) and *in situ* nick-translation assay which exploit the presence of fragmented or nicked DNA within the apoptoic nucleus. This provides a substrate for the addition of labelled nucleotide by terminal transferase (TUNEL) or DNA polymerase (nick-translation). These only detect intermediate and late stages of apoptosis strand breaks prior to DNA fragmentation but again is not specific for apoptosis.

Other methods of distinguishing apoptosis from necrosis include characterisation of intracellular changes using fluorescent probes. These include monitoring swelling and lysis of the cell following mitochondrial damage using Rhodamine-123 retention in apoptosis whereas in necrosis it is not retained. Similarly uptake of the fluorescent stain acridine orange depends on an active ATP-dependent lysosomal proton pump which also is preserved in apoptotic but not in necrotic cells. Typically the cell membrane is permeable to the uncharged form of the dye, but once inside the cells the dye becomes protonated and becomes entrapped and fluoresces red.

There are also intracellular and membrane changes associated with apoptosis (Mower et.al.1994). Apoptotic cells have a low DNA and protein content which is reflected in changes in light scatter: PI positive, Hoechst 33342 positive cells have a reduced forward scatter (size) and slightly increased side scatter (granularity) by flow cytometry. Apoptotic cells may also be isolated by density gradients. These cells also typically show reduced a DNA fluorescence (Telford et.al.1991).

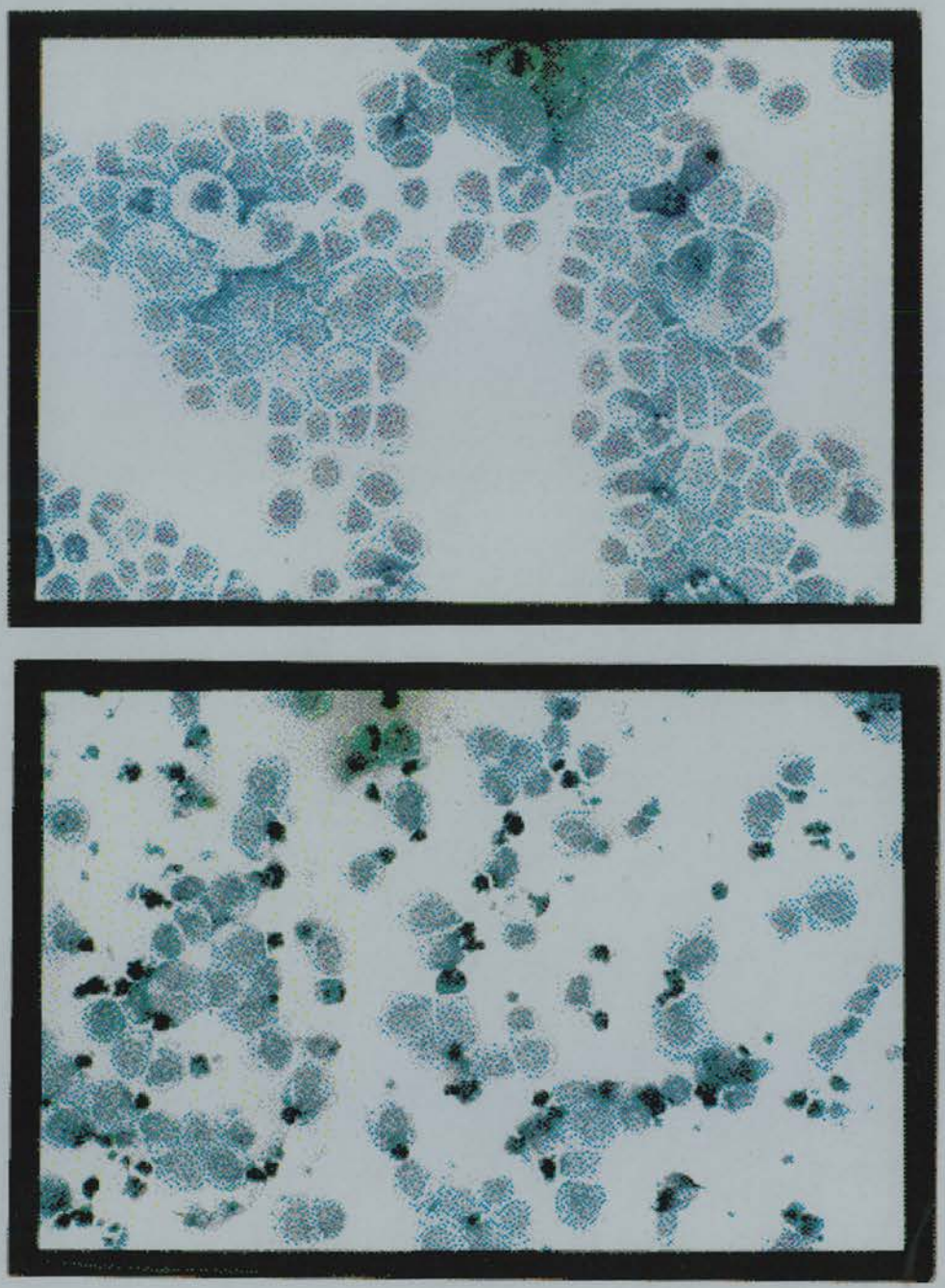
In parallel with these observations the apoptotic membranes express markers such as the thrombospondin receptor and undergo spatial transformation such that inner surface and associated phospholipid moieties become externalised (Fadok et.al.1992). Phosphatidylserine, a negatively charged membrane phospholipid, is thought to be one of many potential signals for signalling the removal of apoptotic cells by phagocytes and can be readily detected by avid binding to Annexin V (Andree et.al.1990). The Annexin V protein is a 320 amino-acid, 36kDa protein which is readliy conjugated to fluorochromes allowing rapid identification of apoptotic cells in combination with

immuno-phenotyping (Koopman et.al.1994, Vermes et. al.1995).

Considerable effort was spent in establishing this reliable and sensitive assay for the detection of small numbers of apoptotic cells. Therefore agents known to effect apoptosis were used *in vitro* to monitor changes in phosphatidyl-serine expression in a dynamic fashion. This would then enable monitoring of the effectiveness of antisense bcl-2 therapy at inducing apoptosis in acute myeloid leukaemia cells. If antisense effects were found on leukaemic cells it was proposed to use this assay to compare the relative effectiveness of different treatment strategies.

Normal peripheral blood mononuclear cells (PBMNC) were used as a control mixed cell population. Cells from AML patients and continuosly growing cell lines KG1-a (acute myeloid leukaemia) and SUDHL-6 (B cell lymphoma) were prepared in a similar manner. Cells were then resuspended in complete medium (IMDM with 10% FCS) at 2 x 10^6 cells per ml and incubated with either 50μM cycloheximide (CHX to inhibit *de novo* protein synthesis through ribosomal peptidyl transferase inhibition) or 4μM actinomycin D (ActD intercalates DNA and inhibits *de novo* RNA transcription but not protein synthesis from existing RNA) to induce apoptosis. Cytocentrifuge preparations of 10^5 cells were stained by May-Grunwald Giemsa staining and apoptosis was confirmed by evidence of cellular shrinkage and nuclear condensation (**Figure 6**).

Aliquots of cells were extracted at 24 hour intervals and examined by histology for evidence of apoptosis. Similarly cells were washed and stained with 1μg/ml human recombinant Annexin V-FITC (BioWhittaker, Berks, UK) in Annexin buffer (10mM HEPES, 150mM NaCl, 5mM KCl, 1mM MgCl, 2mM CaCl) to detect phosphatidyl-serine. Propidium iodide staining (Sigma, UK) was used at (50μg/ml) as evidence of membrane disruption. Cells were analysed by standard flow-cytometric techniques using a FACScan and CellQuest™ software (Becton Dickinson, Oxford, UK) data is presented as bivariate dot plots of Annexin and PI expression. Cells were also studied under standard ultraviolet (UV) fluorescence microscopy for evidence of membrane-bound Annexin V-FITC and for propidium iodide staining of nuclear material.



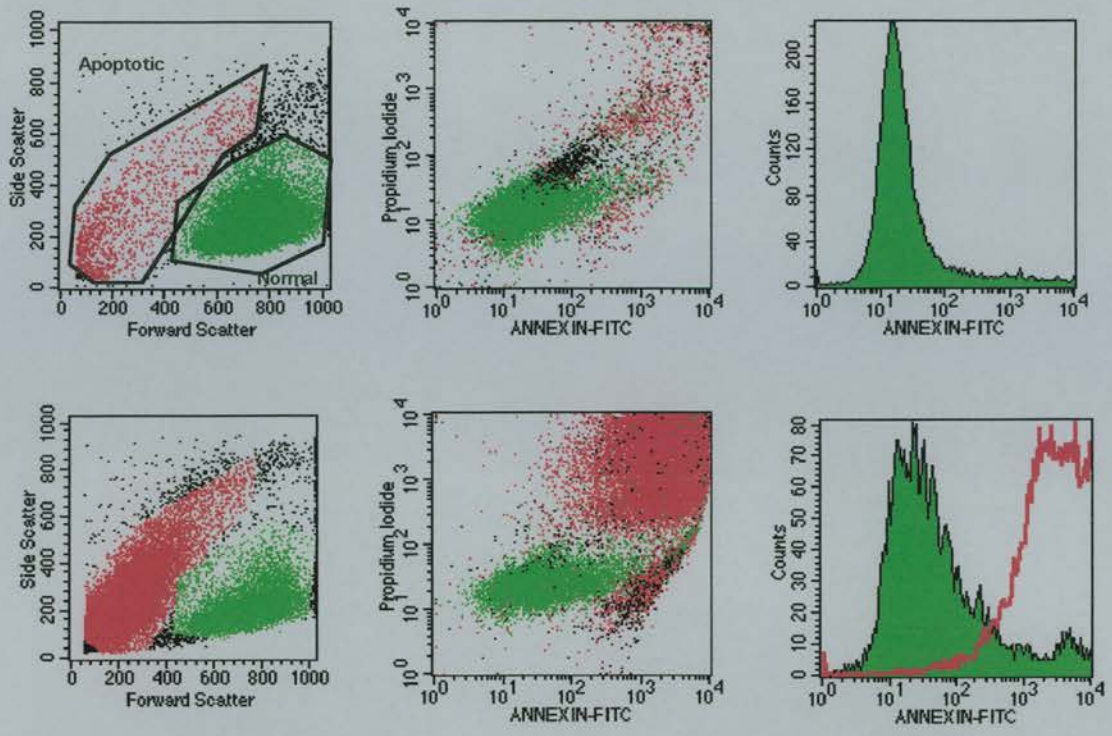
(**Figure 6**). Top: Normal morphology of KG1-a cells.
Bottom: Cells treated with 4μM Actinomycin D for 24 hours showing features of apoptosis. May-Grunwald-Giemsa (x200).

Freshly isolated cells typically had normal morphology and light scattering characteristics (**Figure 6a**) and showed very little (<20%) positivity for Annexin V. Propidium iodide and trypan blue staining both indicated greater than 95% viability. However, of cells treated with cytotoxic agents, those incubated with actinomycin D showed features most typical of apoptosis. Cells had a characteristic reduced volume and condensed nucleii (**Figure.6b**).

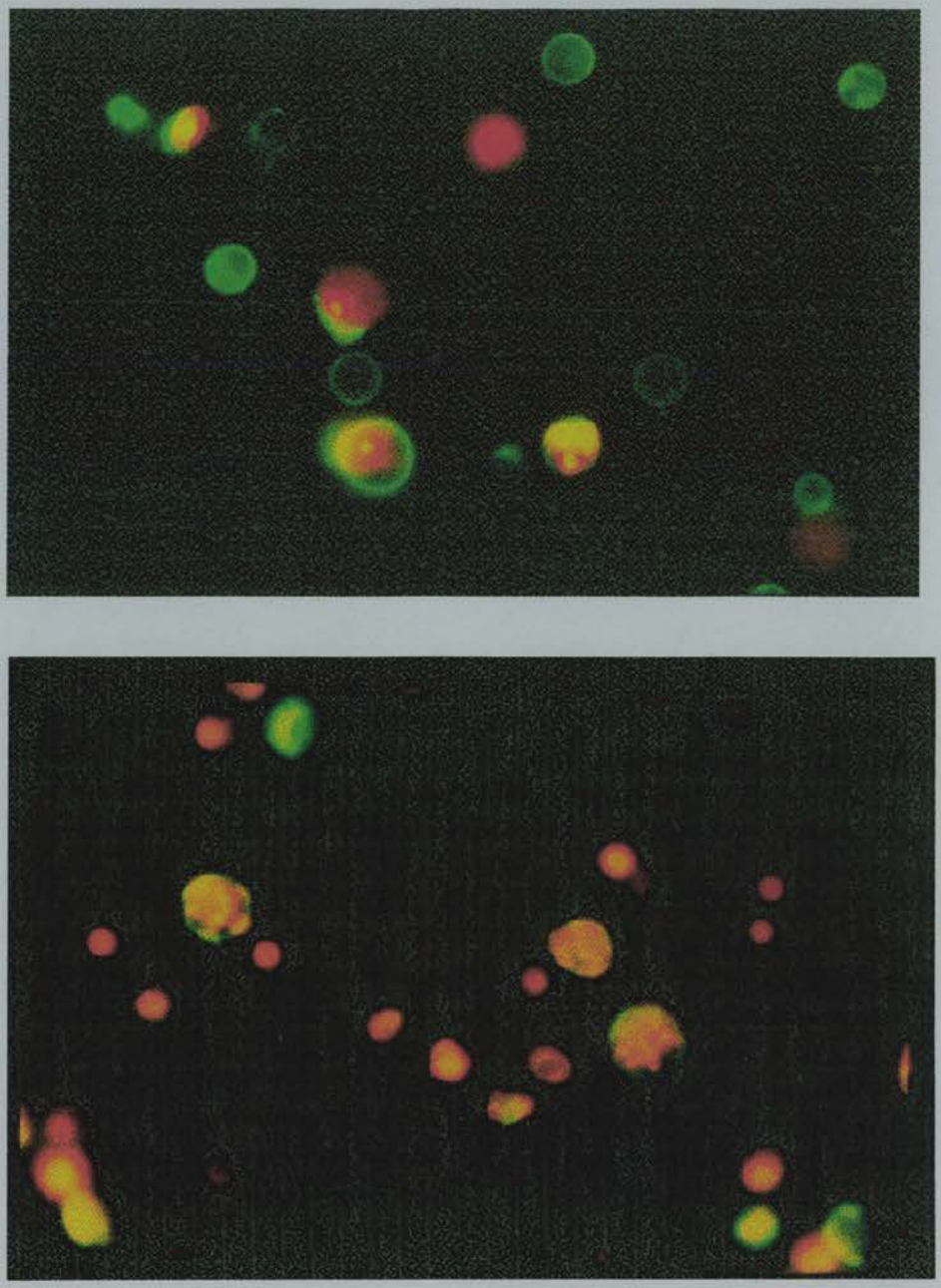
Early changes in light scattering described a separate sub-population of cells formed with low forward scatter (FSC) and elevated side scatter (SSC) with a simultaneous increase in the Annexin V positive population. At 24 hours viability remained greater than 85% indicating membrane integrity (**Figure 7**) remained although by this stage levels of Annexin V-FITC staining had become markedly elevated on most cells (>85%). As treatment progressed beyond 48 hours cell membranes became disrupted and were no longer able to exclude propidium iodide and in many cases there was distinct loss of normal nuclear architecture (**Figure 8**). At this stage Annexin V and propidium iodide staining became intense making it difficult to differentiate between necrotic and apoptotic cells. However necrotic cells typically swell rapidly and burst, leaking contents into the surrounding milieu provoking the release of pro-inflammatiory mediators and are therefore rapidly engulfed by phagocytic cells. It should be noted however that late stage apoptotic cells may undergo a secondary necrosis possibly as a mechanism to recruit further phagocytic cells to the site.

Quantitation of apoptosis by many other existing methods can be time consuming and highly inefficient since few lend themselves to both high throughput and semi-automation whilst providing enough detailed information regarding cellular phenotype, morphology and apoptotic status. Furthermore many depend on lysed or fixed cell populations which similarly reduces the amount of information which can be collected about this dynamic process. For example separation of high from low molecular weight DNA (Wyllie, 1980; Wyllie et.al.1980) using centrifugation and electrophoresis are both time-consuming and depend on lysed cells and presence of DNA fragmentation. Changes in cell morphology eg. chromatin condensation using electron microscopy (EM), phase contrast microscopy (Wyllie et.al.1984) or fluorescence microscopy using DNA binding dyes (Cohen et.al.1992) tend to use fixed, isolated, non-viable cells. Fluorescent histograms demonstrating reduced apoptotic DNA similarly correspond well to classical definitions of apoptotic cells.

Murine B lymphocytes have three distinct stages of apoptosis only the later stages of which can be detected using existing methodology: Stage 1. early apoptosis is associated with decreased cell size and altered plasma membrane organisation; Stage 2. intermediate stage involves DNA cleavage; Stage 3. permeabilisastion of the plasma membrane. Alterations in apoptotic cell fluorescence correlates with reductions in cell size (FSC) (Mangan et.al.1991; Dive et.al.1998; Ormerod et.al.1993). Stage 2 apoptosis has been detected using combinations of fluorescent DNA binding dyes. 7-AAD alone as a marker for apoptosis measures changes in size in combination with fluorescence which correlates well with other techniques (Philpott et.al.1996). 7-AAD may also be used in conjunction with immuno-phenotyping by flow cytometry and requires far fewer cells per assay.



(**Figure 7**). Bi-variate flow cytometric dot plots and histograms showing the increase in Annexin-V positivity as KG1-a cells become spontaneously apoptotic. Membranes lose integrity and become permeable to vital stains such as propidium iodide.



(**Figure 8**). Top apoptotic cells stained with Annexin V-FITC and propidium iodide at 24 hours. Cells at 48 hours demonstrating progressive loss of membrane integrity and nuclear architecture

Apoptotic cells also undergo a variety of membrane changes including expression of thrombospondin binding sites (Pytela et.al.1985), loss of sialic acid residues (Savill et.al.1993) and exposure of phosphatidyl serine (Fadok et.al.1992) resulting from a loss of normal membrane assymetry (Mower et.al.1994). This is an important mechanism in the recognition and removal of apoptotic cells by macrophages (Fadok et.al.1992). Annexin V has been shown to bind negatively charged phospholipids (Andree et.al.1990) in a calcium-dependent fashion. Apoptotic cells become Annexin V positive after condensation of the nucleus occurs but before the membrane becomes permeable to vital stains. Early light-scatter changes described a separate sub-population of cells formed with low FSC and high SSC which identifies the increase in cell density cauised by water loss, with a simultaneous increase in Annexin V +ve population. At later time points both single Annexin +ve and Annexin / PI +ve cells were found and by later stages all cells are double positive. All cells with positive nuclear condensation were also found to be Annexin positive, whereas none of the euchromatic nucleus were Annexin positive . Occasionally a cell with only DNA condensation at the periphery of the nucleus (chromatin margination) was found. These cells represent the early stages of apoptosis and stain only weakly for Annexin. Annexin staining thus coincided with the appearance of nuclear condensation in apoptotic cells.

DNA fragmentation into oligosomal bands has been shown to be present only in sorted Annexin V staining cells. At later time points however, oligosomal band intensity decreased, possibly due to further DNA degradation, whereas the level of Annexin staining in cells with condensed a nucleus increased. Morphologically evident apoptosis however, is not always followed by DNA fragmentation (Cohen et.al.1992; Falcieri et.al.1993) though inter-nucleosomal cleavage by endonucleases is often used to detect apoptosis.

In this thesis apoptotic cells were stained with Annexin V-FITC followed by membrane permeabilisation and PI staining of DNA content. This revealed that the two stains identified similar populations, albeit with Annexin V describing a higher percentage apoptotic cells because of the membrane changes preceding DNA changes.

In conclusion phosphatidyl serine expression, and hence Annexin V-FITC positivity, correlates well with many of the standard observations of apoptosis determination eg. TUNEL, Hoechst 33342-EtBr staining and provides a sensitive and reliable detection system. The combination of reversible high affinity binding (approx 10^{-10}M) and a potentially large number of phosphatidyl serine sites (approximately 10^6) on the surface of apoptotic cells allows a sensitive process of identifying apoptotic cells. Early stage apoptotic cells may be readily identified and isolated on the basis of their

phosphatidyl serine expression and may be collected for further studies using flow or immunomagnetic sorting. Early apoptotic cells may be differentiated from late apoptotic and necrotic cells on the basis of their light scatter and Annexin-propidium iodide staining patterns. In conjunction with fluorescent antibodies to cell-surface markers Annexin V-FITC may be used to identify sub-populations of cells undergoing apoptosis in a dynamic system. This may have important considerations in clinical applications where the effects of cytotoxic agents on discrete sub-populations of cells such as tumours within a larger population of heterogeneous cells must be considered.

This Annexin V-FITC staining method was therefore routinely used to quantify apoptotic cell populations and in the determination of the functional effectiveness of the antisense therapy at killing leukaemic cells. Propidium iodide staining of DNA content was used to identify stages in the cell cycle which were sensitive to drug and as an independent confirmation of apoptosis.

Bcl-2 expression

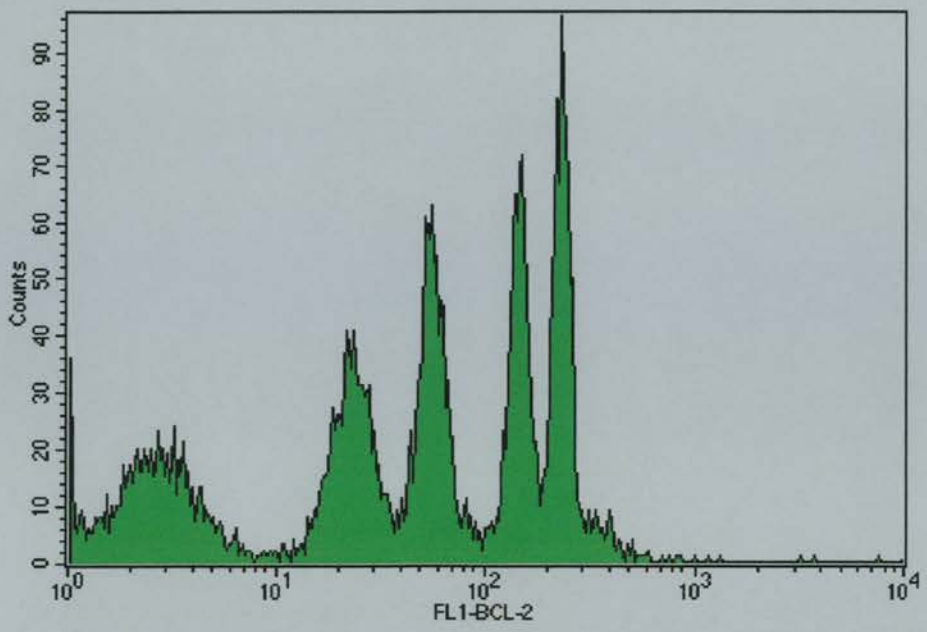
Normal blood and bone marrow

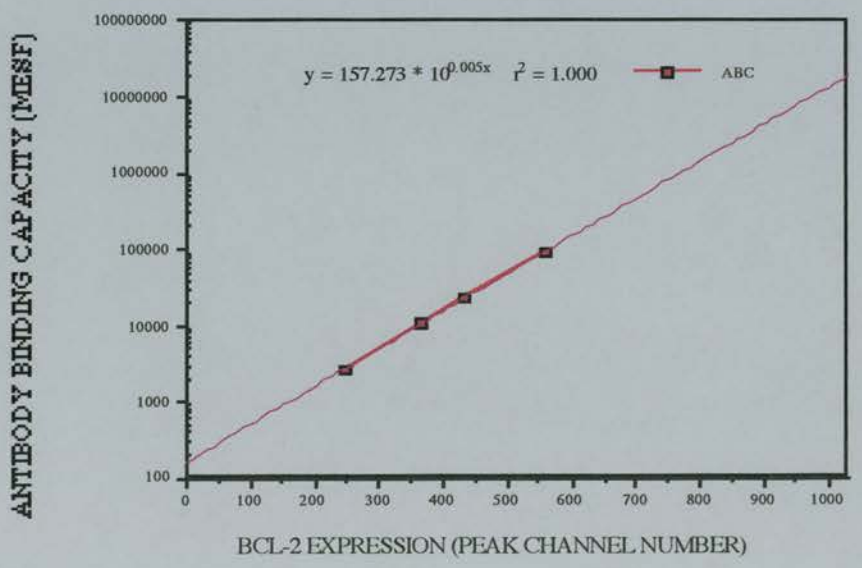
To determine whether patients with AML had elevated levels of bcl-2 protein expression a sensitive technique was required for quantitation. It was felt that densitometric scans of Western blots would not provide the sensitivity required nor the ability to discriminate sub-populations of cells within heterogeneous mixtures such as bone marrow or peripheral blood. Therefore a quantitative flow-cytometric assay was developed using immunophenotyping followed by cell permeabilisation and staining with anti-bcl-2 monoclonal antibody (Clone-124 DAKO Ltd,UK). The assay uses a measurement known as the mean equivalent of soluble fluorochrome (MESF) which is used to construct a calibration of fluorescence intensity.

MESF is the relative channel number (RCN) for log amplifiers (RCN = peak channel number x 10^{nd} where nd = neutral density of filter normally has a value of zero) (Macey, 1994).

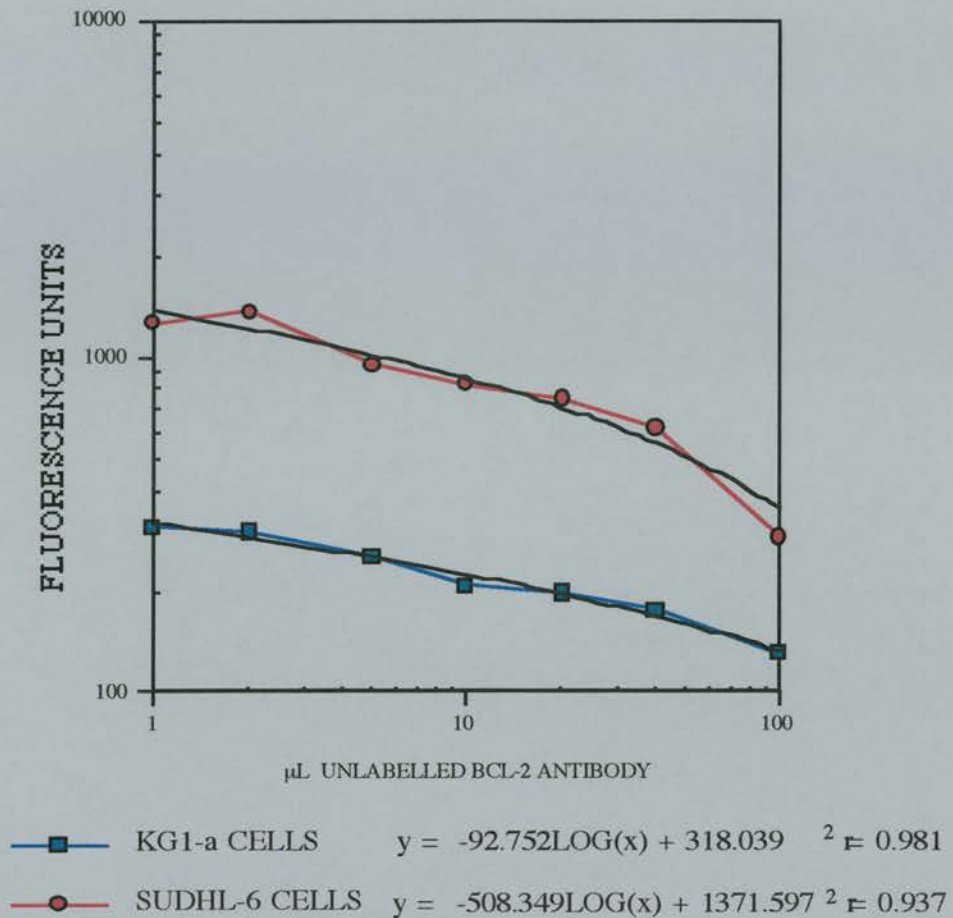
The Quantum Simply Cellular Kit (Sigma, UK) uses a panel of five beads; one blank and four standards. These beads are coated with a fixed and absolute binding capacity for mouse antibodies and are used to construct a calibration curve for the antibody binding capacity (ABC) versus fluorescence signal intensity in channel numbers (**Figure 9a**). Thus given a relative fluorescence intensity the ABC (or MESF) can be given and is independent of the instrumentation the data is acquired on and the software used to analyse it. Data from different laboratories can thus be directly compared. In this study this was done for bcl-2, CD34, IgG antisera

The calibration curve for bcl-2 antibody is shown below in (**Figure 9b**).


Counts
0
10
20
30
40
50
60
70
80
90
10^0
10^1
10^2
10^3
10^4
FL1-BCL-2


ANTIBODY BINDING CAPACITY (MESF)
100000000
10000000
1000000
100000
10000
1000
100
y = 157.273 * $10^{0.005x}$ r^2 = 1.000
ABC
0
100
200
300
400
500
600
700
800
900
1000
BCL-2 EXPRESSION (PEAK CHANNEL NUMBER)

SPECIFICITY OF BCL-2 ANTIBODY



(**Figure 9c**). Demonstrating the specificity of bcl-2 staining in cells following permeabilisation.

The specificity of the bcl-2 antibody (clone 124 DAKO, UK) was confirmed using a competition experiment. Increasing amounts of unlabelled antibody were co-incubated with the FITC labelled bcl-2 antibody and added to a fixed amount (10^6 cells) of either KG1-a (myeloid leukaemia cells) known to express high levels of bcl-2 or SUDHl-6 cells with a t(14;18) translocation, thus with deregulated elevated bcl-2 levels. It can be seen from the plot that there is direct competition between the labelled and unlabelled antisera demonstrating specificity of staining (**Figure 9c**).

Lymphocytes have been shown to express intense bcl-2 staining, monocytes were weaker and neutrophils essentially bcl-2 negative (Iwai et.al.1994). Resting peripheral blood lymphocytes (PBL) expressed both bcl-2 protein and message, whereas lymphoblasts in mitogen-stimulated cultures lost bcl-2 protein and became apoptotic despite expressing high levels of bcl-2 mRNA. This may be the result of post-transcriptional regulation of the bcl-2 gene or post-translational modification of the protein (Akagi et.al.1994). More than 80% normal T and B cells were found to be bcl-2 positive with T and B cells having similar levels (Aiello et.al.1992). Differences in absolute levels between different studies may be related to the cellular processing method. Bcl-2 protein was detected in fresh PBL but levels decreased on storage overnight (Reed et.al.1992). It then underwent a marked increase upon stimulation of cells by mitogens and lectins and cytokines. Similarly bcl-2 was found to be present in freshly harvested blood but not in buffy coats independent of the anticoagulant used for collection.

Results found in this thesis therefore compare favourably with those found by other authors both in normal and malignant cell types and values fall into the expected ranges. All samples were processed for bcl-2 expression on fresh samples within 2 hours of harvesting where possible. 70% methanol in PBS was used as the fixative and permeabilisation agent since it had been shown that methanol or ethanol (70% in PBS) both gave similar levels of fluorescence intensity (Steck et.al.1995). The methanol fixation method was selected because it allowed the simultaneous determination of cell cycle phases (Slezak et.al.1989) with bcl-2 and immunophenotype determination.

Peripheral blood T and B cells were found to express similar amounts of bcl-2 protein (29-37 x 10^3 MESF per cell), bone marrow monocyte precursors had lower amounts (21 x 10^3 MESF) while peripheral blood monocytes had lower levels again (15 x 10^3 MESF) (Porwit-McDonald et.al.1995). These levels are somewhat lower than levels found in this thesis and the patterns are different although CD3 and CD19+ lymphocytes were also found to have equal levels. In these volunteers normal peripheral blood samples CD3+ T lymphocytes and CD19+ B lymphocytes were

found to have similar values (n= 9 CD3+ 79,074 +/- 29,608 MESF; CD19+ 72,691 +/- 20,428 MESF), monocytes had similar levels but with a greater range 118,632 +/- 42,867 MESF (**Figure 10**). The reason for this discrepancy is unclear but may be related to processing. Reed (1992) found that storage of cells for extended periods resulted in a reduction in bcl-2 levels and this may occur in different cell types at different rates.

Bone marrow CD34 positive blasts and myeloblasts have have twice the levels of lymphocytes 51 x 10^3 MESF. CD34+, CD10+ lymphoblasts have low levels of bcl-2 (8-10 x 10^3 MESF) and CD34-CD10+ lymphoblasts have even less <5 x 10^3 MESF (Porwit-McDonald et.al.1995). In this study CD34+ cells from normal bone marrow or peripheral blood stem-cell donors had comparable levels (73,822 +/- 32,498 MESF) to lymphocytes (**Figure 10**).

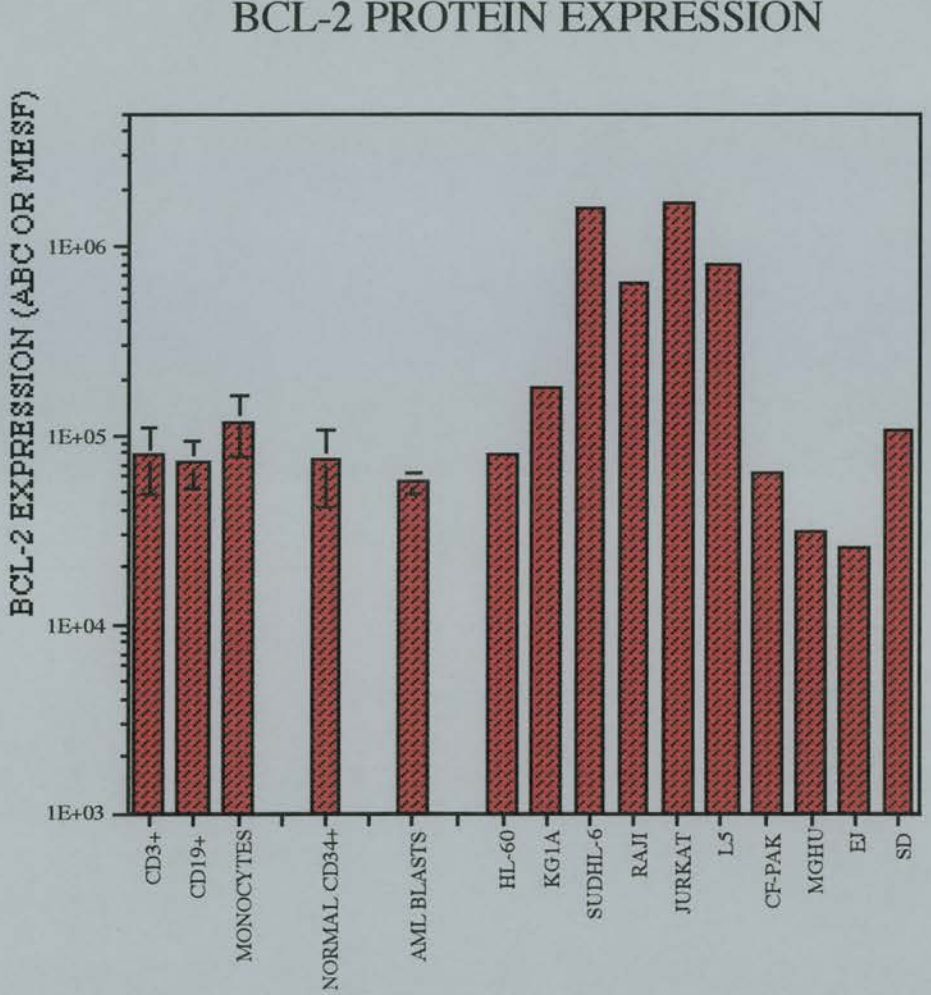
AML cells

Other authors have noted an association between bcl-2 expression and response to chemotherapy and found that patients with more than 20% bcl-2 positive blasts related to a 29% complete remission (CR) rate whereas less than 20% bcl-2 positive blasts equated to 85% complete remission (Ganser et.al.1994). Bcl-2 expression was found to be highest in the monocytic M4 and M5 AML patients and was correlated to CD34 expression. High levels of bcl-2 protein in undifferentiated cells may lead to prolonged lifespan. Bcl-2 levels decrease in AML with granulocytic and monocytic differentiation with AML M1 and M2 expressing significantly higher levels than M3 or M4 or 5. Small undifferentiated blasts which were HLA-class II ^{dim} have higher bcl-2 levels than large differentiated blasts which were class II ^{bright}. AML M1 were found to have 40% bcl-2 positive blasts, M2-50%, M3-22%, M4-19%, M5-15% with an overall average of 25 x 10^3 MESF for all groups of AML (Porwit-McDonald et.al.1995).

Similarly of 82 newly diagnosed AML samples bcl-2 content was measured and found that a mean of 23% were positive. The percentage bcl-2 positive blasts was found to be higher in M4 and M5 sub-types and those with highest blast cell counts. Bcl-2 expression was correlated with CD34 expression and high levels associated with poor complete remission and shorter survival. KG1-a cells (an undifferentiated AML M0 AML cell line) were 97% bcl-2 positive and HL-60 (promyelocytic leukaemia cell line) cells 85% positive though their intensity was lower than KG1-a (Delia et.al.1992). Bcl-2 percentage positive cells were highest in monoblastic M4 and M5 and lowest in M3 promyelocytic AML. Another study found that M0 were 15% bcl-2 positive, M1-25%, M2-14%, M3-8%, M4-35%, M5-29%, M6-35%. Normal normal foetal bone-marrow has the highest levels but bcl-2 was found in mature myeloid cells up to the

promyelocyte and erythroblast stages. Differentiated HL-60 had a much reduced bcl-2 levels and it was suggested a high bcl-2 expression in AML is a consequence of the absence of differentiation, rather than deregulated expression (Campos et.al.1993)

As a group the AML leukaemic blasts cell bcl-2 MESF in this thesis was 56,208 +/- 6879 MESF n=55 a level which was similar to normal CD34+ cells and lymphocytes (**Figure 10**). However there were individual patients within the group with a higher bcl-2 content. It was not possible to further identify the disease classification of these AML patients or their subsequent disease progress as the clinical information was not made available. The highest levels of bcl-2 were found in the hematologic cell lines. Those with the t(14;18) translocation having the highest levels (SUDHL-6 cells 1,594,338 MESF). This is not surprising since these cells have deregulated bcl-2 levels with this type of translocation. Other haematologic cells similarly have elevated levels of bcl-2 protein, the mechanism by which this arises is not clear, though may be related to altered growth factor expression. KG1-a (AML cell line) had 178,620 MESF, HL-60 (promyelocytic leukaemia) cells had a MESF of 78,837, Raji (Burkitts lymphoma) 642,571 MESF, Jurkat (T cell lymphoma) 1,677,585 MESF. These levels were found to be higher than those associated with carcinoma cell lines MGH-U-30,620 MESF and EJ-25,040 MESF, CF-PAK-64,305 MESF, though in one cell line SD (104,662 MESF) the levels were similar to KG1-a levels (**Figure 10**). Predictably in KG1-a cells and in blast cells from AML patients associated with light scatter characteristic of apoptotic cells showed a marked reduction in bcl-2 levels such that a bi-phasic population of bcl-2 expression was often seen (**Figure 30**). Healthy cells had approximately an order of magnitude more bcl-2 protein than apoptotic cells. Thus apoptotic cells could now be identified either by their bcl-2 expression, Annexin V positivity, light scatter characteristics or DNA histogram depending on which was more approriate for the experiment. This feature has similarly been observed by other authors (Bradbury et.al.1997; Bradbury et.al.1994) but was not commented upon.



(**Figure 10**). Levels of bcl-2 protein expression in differing cell types as assesed by flow cytometry. MESF was determined from the calibration curve constructed using the Quantum Simply Cellular™ calibration kit (Figure 9b).

Colony formation as a determinant of Residual Disease

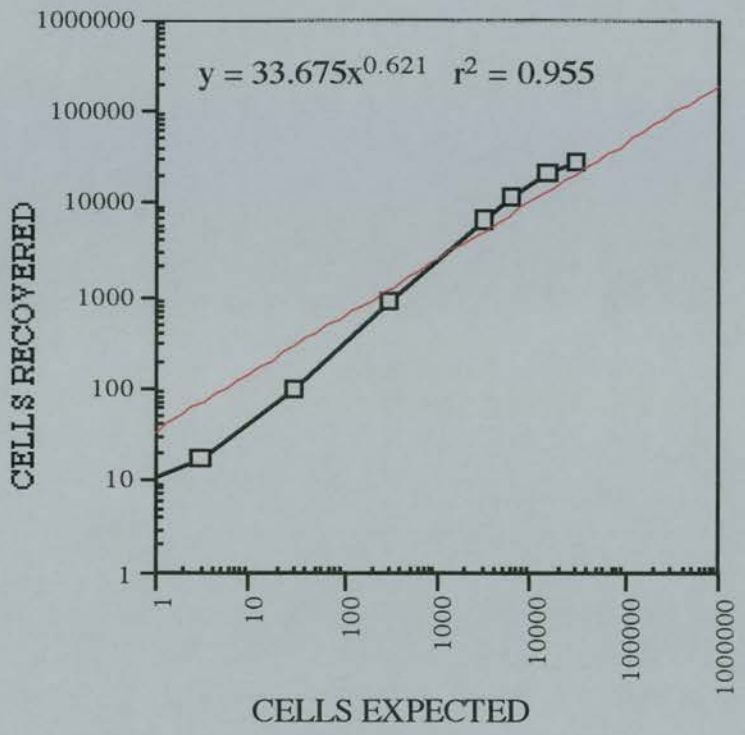
The sensitivity of detection of tumour cells is greatest with the polymerase chain reaction (PCR) and multiparameter flow cytometry with levels of sensitivity approaching one tumour cell in 10^4 (flow cytometry), or 10^5 (PCR) normal cells with a realistic separation at a level of 10^{-4}-10^{-5} cells by PCR (Campana et.al.1995; Negrin et.al.1991).

In this thesis the theoretical limits of tumour cell detection by flow cytometry were determined using the stable lipophilic membrane dye PKH-2. Following optimisation of staining conditions this green fluorescent dye is incorporated into cell membranes where it remains stable for up to 10 days (Horan et.al.1990). Serial log dilutions of labelled KG1-a cells stained with 1μM PKH-2 were seeded into buffy coat cells. 30,000 cells were counted by flow cytometry and and the number of positive cells recorded. A plot of expected versus recovered positive cells confirmed that the expected sensitivity of flow-cytometric detection of tumour cells is is approximately one cell in 10^4 (**Figure 11a**).

PCR and immunocytochemical detection of tumour cells however do not detect tumour viability or clonogenicity. Cell culture allows approximately a 2 log increase in the number of cells representing 7-8 cell divisions (2^8=256). Culture systems must however provide appropriate ligands and growth factors to ensure appropriate selective conditions for tumour cell survival and growth (Sharp et.al.1992; Sharp et.al.1995; Sharp et.al.1996). Growth patterns of AML cells in semi-solid culture systems have been classified by into four growth patterns and the models related to disease prognosis (Reilly et.al.1989a; Lowenberg et.al.1993). They defined a parameter known as the autostimulatory index (ASI) to classify four patterns of cell growth. ASI is colony growth in the absence of conditioned medium (CM) divided by the number of colonies grown in conditioned medium from the bladder cancer cell line 5637. This cell line has been shown to secrete growth factors (IL-1, IL-3, IL-6, GM-CSF) which promote leukaemic colony growth *in vitro* (Gabrilove et.al.1986).

Patients with AML blasts defined as group 1 do not grow under any of the conditions; group 2 patient blast cells are colony-stimulating factor (CSF)-dependent and have an ASI of less than 0.1; group 3 AML patients have partially autonomous growth and have an ASI of between 0.1-0.8. Blast cell growth in these patients can be further stimulated by addition of exogenous growth factors; group 4 patients exhibit fully autonomous gowth and are not stimulated further by growth-factor addition and have an ASI greater than 0.8.

SENSITIVITY OF FLOW CYTOMETRIC CELL DETECTION



(**Figure 11a**). Demonstrating the levels of sensitivity of detection of rare cells by flow cytometry. KG1-a cells labelled with PKH-2 were seeded into buffy coats and the number of positive cells detected versus the expected count was plotted.

Blast-cell conditioned-medium from autonomous growing blasts has been shown to enhance normal CFU-GM formation. Similarly conditioned medium from cultured AML patient blast cells stimulated the growth of other AML blasts except those from patients in group 4, the conditioned medium from which showed no autostimulatory activity on other cells. This growth promoting activity is though to be due to release of GM-CSF (regulated by IL-1β) acting in an autocrine and paracrine fashion on the blast cells to promote cell proliferation. Anti-GM-CSF antibody however failed to inhibit group 4 blasts autonomous growth even though they did secrete GM-CSF therefore this growth factor was thought to act in a direct intracellular signalling manner. Group 3 cells can be made to grow autonomously if the cell density is increased (Reilly et.al.1989a).

Autonomous growth of AML blast cells *in vitro* is also associated with higher bcl-2 levels. Antibodies to GM-CSF were able to down regulate bcl-2 levels, and exogenously added GM-CSF in dependent cells, caused an increase in bcl-2 protein expression. 78-90% of CD34+Lin- cells expressed bcl-2, however very early haemopoietic cells (CD34+, Lin-, 38+) were bcl-2 negative but expressed bcl-xl. CD34+ normal bone marrow had greater than 75 x 10^3 MESF bcl-2. Whilst CD34+ normal bone marrow cells express bcl-2; CD34 positive AML cells express higher levels of bcl-2 than CD34 negative AML cells. Bradbury found that CD34+ AML MESF expressed 43.6 +/- 3.3 x10^3 MESF bcl-2; CD34- AML expressed 31.7 +/- 3.7 x 10^3 MESF bcl-2. CD34+ blasts had 65-107 x 10^3 MESF bcl-2 whereas CD34- blasts from the same patient were 33-82 x10^3 MESF bcl-2. AML blasts express 11.1-99.9 x 10^3 MESF bcl-2. Growth factor dependent cells (groups 2 and 3) were 19.3+/- 2.4 x 10^3 MESF bcl-2 (rising to 27.9+/- 4.4 x 10^3 upon addition of GM-CSF); CSF-independent cells had a higher level 40.9+/- 3.6 x 10^3 MESF bcl-2. No detectable increase in apoptosis was seen with the addition of an anti-GM-CSF antibody even though there was a reduction in bcl-2 expression (51.4+/- 8.9 decreasing to 38.5+/- 7.4 x 10^3 MESF bcl-2 upon antibody addition). The presence of high levels of bcl-2 may therefore maintain cell viability in the absence of growth factors (Bradbury et.al.1997).

Bcl-2 levels in KG1-a cells and other cell types studied in this thesis (lymphoma and AML patients, SUDHL-6 cells, Raji) appeared to be related to stages in the cell cycle. Simultaneous measurement of DNA and bcl-2 content showed that larger blast-like cells appeared to be in the G_2-S-M phases of the cell cycle and expressed elevated (2-fold) levels of bcl-2 over cell in the G_0-G_1 regions. Other authors have however found no association between bcl-2 content and cell cycle status.

In order to determine the detection limits of the colony assay a control system was established. Serial log dilutions of KG1-a (acute myeloid leukaemia) cells were seeded into 0.8% methylcellulose BCCA. The number of colonies were counted at both 5 and 16 days to see if sensitivity could be increased by allowing a greater number of cell divisions. The sensitivity was found to be similar in both cases with a cell dilution of 2 x 10^4 cells per ml being an approximate cutoff, beyond which there was no colony formation (**Figure 11b**). This approximates to a maximum of one leukaemic colony forming cell per 200 KG1-a cells seeded.

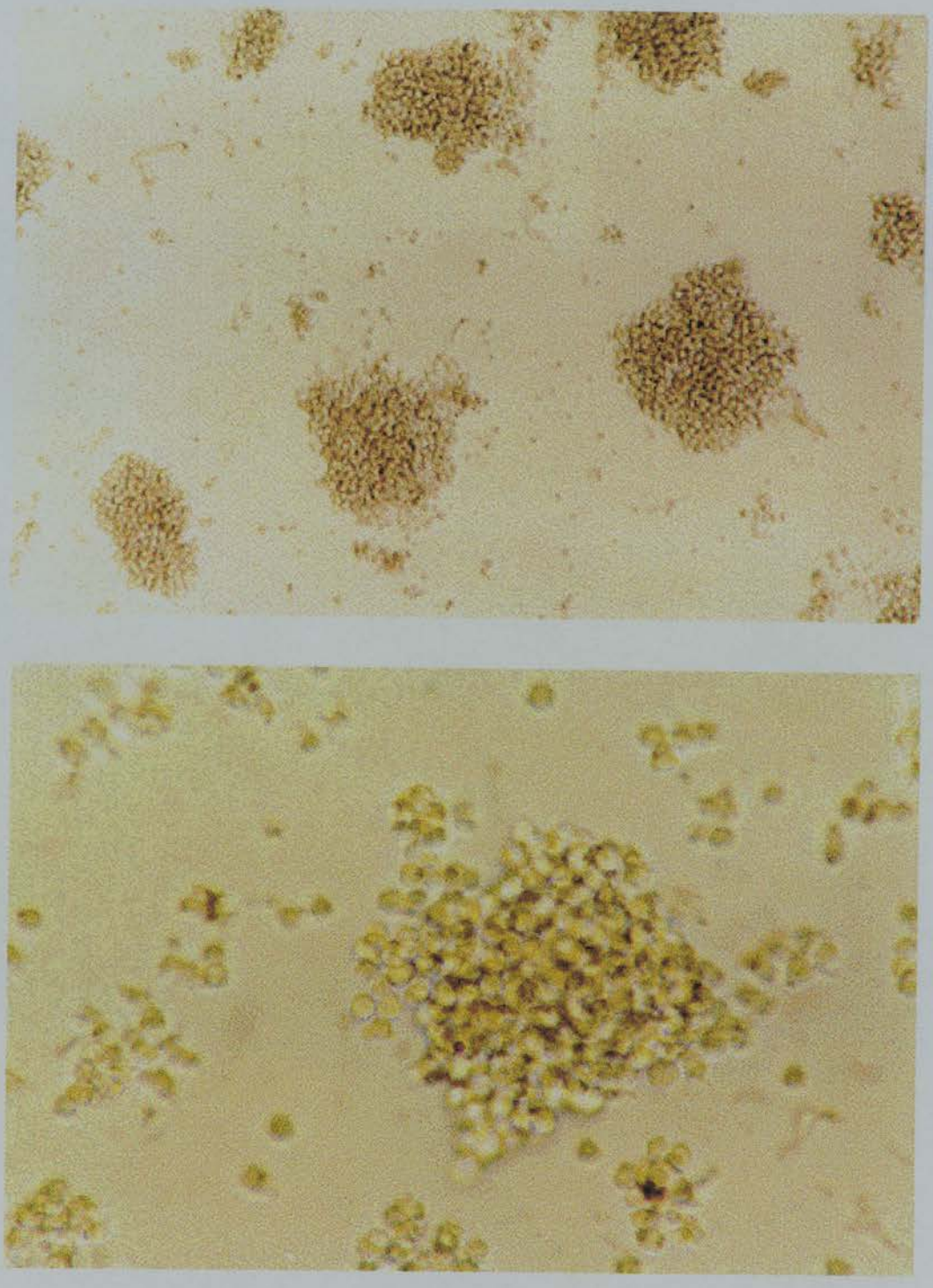
A maximum of 1 cell in 1-200 is able to form a colony, again stressing the unique value of the CFU assay at detecting only replicating cells (Lotem et.al.1993). Though these cells may appear positive by flow cytometry or PCR they may in fact not contribute to disease relapse. Therefore even with the exquisite sensitivity of these approaches the true leukaemia-initiating cell may not be detected. Further isolation and characterisation of these leukaemic colony-initiating cells should lead to more information on the cellular defect(s) which precipitate the leukaemic process. Only then will effective strategies be designed and able to target most effectively these cells rather than the more differentiated highly replicative progeny. Recent studies on the SCID mouse acute myeloid leukaemia-initiating cells have suggested these cells expressed CD34 will be discussed in more detail elsewhere in this thesis (Terpstra et.al.1996; Terpstra et.al.1997; Bonnet et.al.1997).

However an attempt to purify these cells a CD34+ immunomagnetic (MACS™) cell isolation was undertaken. Here CD34+ KG1-a cells were seeded into buffy coats at serial log dilutions and CD34+ cells isolated. However it was found that only a tenfold increase in the number of selected CD34+KG1-a cells could be achieved and not the 10^6-10^7 fold increase as suggested by the manufacturers literature. These selected CD34+ cells were found to have a reduced CD38 expression and lower granularity indicating a less mature phenotype but studies to confirm that these cells were leukaemic CFU-initiating cells were not performed as there were insufficient funds available to repeat the experiment.

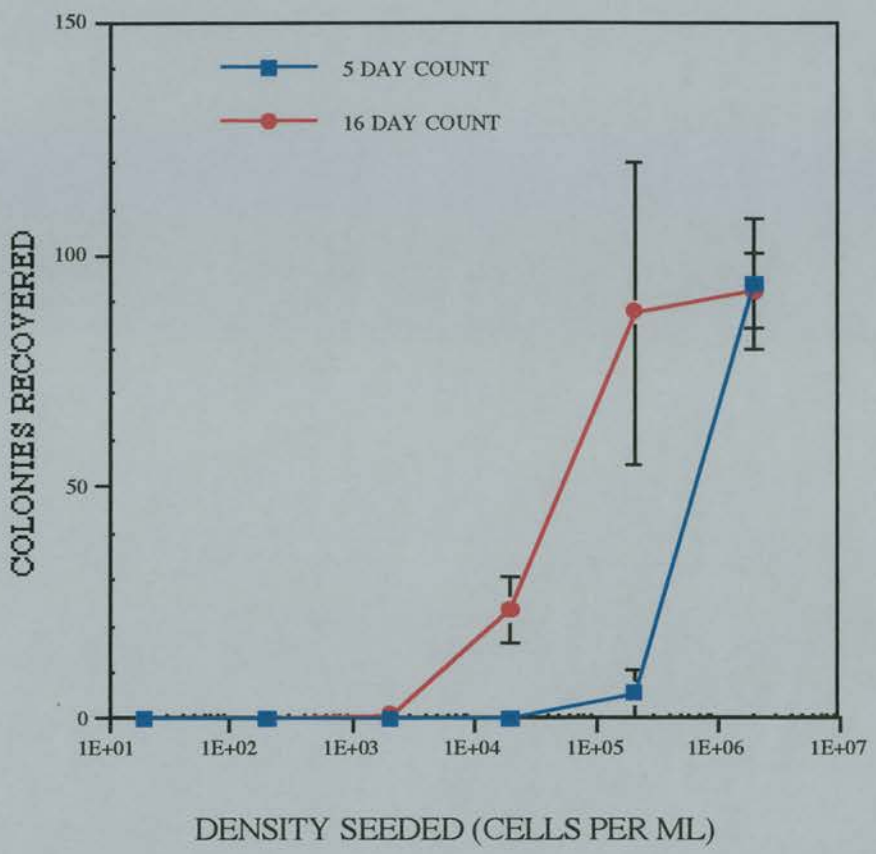
Similarly KG1-a cells were labelled with the stable membrane dye PKH-2 and seeded in serial log dilutions into Jurkat cells with the cells harvested at 24 hour intervals. It was expected that as leukaemic cells divided they should express a two-fold reduction in the PKH-2 dye content with a concomitant doubling of cell numbers.

It was felt that in combination with CD34+ antibody staining, this dye should identify the colony-initiating cells. It was expected that the more proliferative, more mature cells would lose their CD34 positivity, increase in granularity and reduce their PKH-2

content. Leukaemic colony-initiating cells were expected to maintain their CD34 positivity, low granularity and retain a high PKH-2 content. As expected there was a reduction in PKH-2 content in KG1-a cells as they divided but unexpectedly the Jurkat cell line seemed to increase in positivity. It was felt that these cells were phagocytosing labelled KG1-a cells either because of some marker expression (eg. KG1-a cells are known to express Fas and Fas ligand -Dr H Roddie personal communication) following labelling or due to an limited anti-leukaemic effect. In any event the experiment was abandoned.



(**Figure 11b**. Top: Low power magnification (x125) of colony formation from a patie
with AML. Colonies with greater than 20 cells were counted at 7 days.
Bottom: High power magnification (x300) demonstrating the clonality of these cells.



(**Figure 11c**). This graph demonstrates the levels of detection of leukaemic colony-forming cells in methyl-cellulose. The limit appears to be one colony formed at 2000 to 20,000 cells per ml depending on the length of incubation.

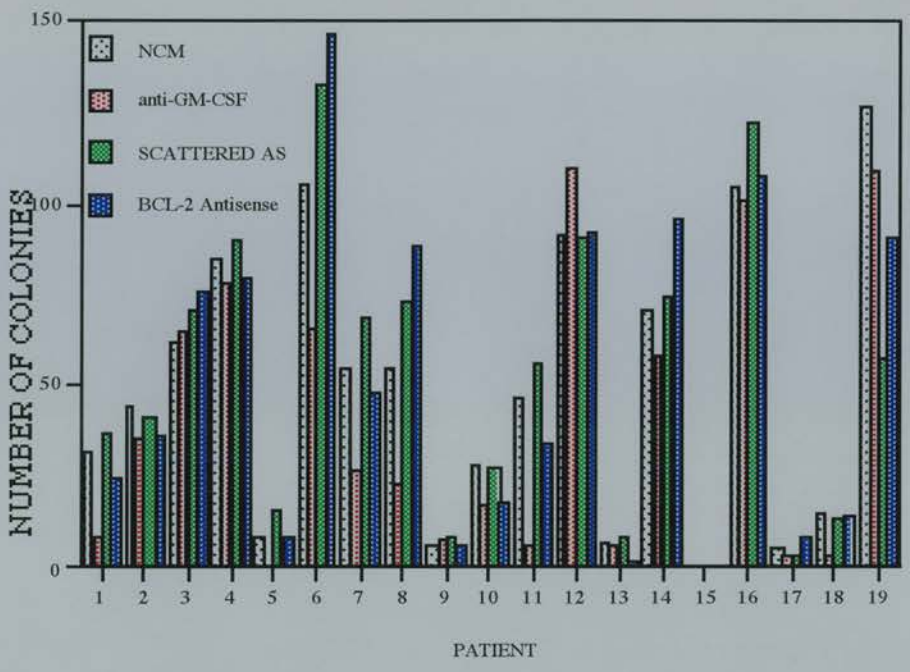
Other authors have suggested that cell-cell contact is required for autonomous AML blast growth. AML blasts were cultured in flat-bottomed wells at 15-500 cells per ml and DNA synthesis was found to be related to cell density. Growth in round-bottomed wells was independent of cell numbers, though physical separation of cells using latex beads abrogated this effect (Reilly et al 1989). Other studies showed most AML patient blasts required exogenous colony-stimulating factors to promote and sustain growth. At least 10^6 blast cells per ml were required for optimal growth, but this effect could in part, be substituted by a portion of irradiated blasts or isolated membranes (Nara et.al.1985). Blast cell conditioned medium from crowded cells was able to enhance the growth of cells more than conditioned medium from non-crowded cells. This presumably arises from the adhesion-dependent autocrine GM-CSF release. Prior culture of blasts in round-bottomed flasks to enhance cell-cell contact for 72 hours gave rise to more colonies than flat bottomed culture alone. Cell-cell contact was capable of stimulating blast cell growth which was decreased by physical separation using latex beads whereas mitomycin c treated blasts increased blast cell growth (Reilly et.al.1989b).

In the study outlined in this thesis 77 AML samples were processed in the blast cell colony assay (BCCA). Of these 18/77 (23.4%) showed partial or complete autonomous growth (**Figure 11d**). This is much less than the 70% patients described by Russell's group (Reilly et.al.1989a; Hunter et.al.1993; Russell et.al.1995) but is similar to the findings of others who described autonomous growth only in a minority of AML patients (Cheng et.al.1988; Young et.al.1988; Metcalf, 1986). When all variables were taken into consideration autonomous growth was found to be the single most important indicator of complete remission (CR) and disease free survival (DFS) (Hunter et.al.1993). The 5 year survival of non-growers was 54.2% as compared to 11.3% survival for autonomous growers. Group 1 & 2 patients had a complete remission rate of 94% as compared to 57% for patients in group 3 & 4 . It was not possible to follow up the course of the disease in patients studied in this thesis since clinical information was not made available on these patients.

As intimated earlier cells which demonstrate autonomous growth in the blast cell colony assay have elevated levels of bcl-2 possibly related to growth-factor mediated tyrosine kinase signalling mechanisms, and may also lack functional retinoblastoma protein (Olsson et.al.1996). The mean MESF bcl-2 per cell in all AML cases was 11-74 $x10^3$ molecules per cell (MESF), the patients in this thesis had a mean of 56 with range of 11-306 x 10^3 MESF. Group 1 & 2 patients were found to have low levels of bcl-2 (19.3 x 10^3 +/- 2.4 molecules per cell). Group 3 & 4 patient blasts on the other hand expressed 40.9 x 10^3 +/- 3.6 molecules per cell. In those patients where it was effective an anti GM-CSF antibody was able to down-regulate bcl-2 from 51.4 +/- 8.9

x 10^3 to 38.5 +/- 7.4 x 10^3 molecules per cell. This was not however associated with induction of apoptosis but did lead to an increased sensitivity to cytotoxic drugs (Lotem et.al.1993). Conversely G-CSF which is used to ameliorate chemotherapy-induced myelosuppression may enhance tumour cell proliferation and has been described to enhance the survival of leukaemic cells in response to the cytotoxic effects of adriamycin and etoposide (Kondo et.al.1994).

Of the patients studied a variable response was seen to an anti-GM-CSF neutralising antibody present at 5μg/ml (sufficient to neutralise more than 250IU/ml GM-CSF). Only 3/18 patients showed any demonstrable reduction in tumour cell growth (**Figure 11d**). A further 8 peripheral-blood progenitor cell harvests from patients demonstrating autonomous growth of blast cells at presentation revealed no autonomous growth in this assay. This could be related to the low density of blast cells in these harvests and and the probability that only one in a hundred cells is likely to be a leukaemic CFU cell. However this pattern of colony inhibition is consistent with the poor level of autonomous cell growth seen in these patients. There was no funding available to perform a CD34 positive immuno-magnetic selection on these samples to see if the sensitivity could be increased. It was not clear which, if any, of the colony-stimulating factors, these blast cells were dependent upon for growth since there was occasional colony formation similar to early CFU-GM formation in samples cultured in the presence of conditioned medium. Indeed many group 4 patients' cells show a poor response to the inhibitory effects of neutralising antibodies leading some researchers to suppose that these may in fact be truncated receptors acting purely in an intracellular environment.



(**Figure 11d**). Peripheral blood and bone marrow samples from AML patients were grown in the blast cell colony assay to look for autonomous growth. 18/77 Patient showed some degree of growth and were treated with a neutralising anti-GM-CSF antibody or bcl-2 or control antisense to purge leukaemic colony-initiating cells.

Antisense bcl-2

Because of the limited availibility of AML samples which demonstrated autonomous growth attention was concentrated on the development of the therapeutic potential of bcl-2 protein modulation. The suitability of bcl-2 as a potential therapeutic target remains promising however, since as indicated, patients with high levels of autonomouus growth and elevated levels of bcl-2 present with a poorer disease prognosis. Strategies such as therapeutic manipulation with monoclonal antibodies to growth factors and their receptors have a limited clinical role because of their poor discrimination between normal and tumour cells and the potential to initiate an anti-antibody response. Therefore other tactics have been adopted in AML including differentiation of blast cells.

ATRA or recombinant colony-stimulating factors may differentiate blast cells or recruit blasts into cycle to make them more responsive to S-phase specific drugs or to induce terminal differentiation of blasts. Other pharmaceutical agents may perform similar functions. For example the promyelocytic leukaemia cell line HL-60 can be differentiated using 1.2% DMSO, plus PMA (10ng/ml) every 72 hours. This induces differentiation to monocyte like cells with a concomitant reduction in bcl-2 protein expression. These differentiated HL-60 cells which have much reduced bcl-2 were found to be in G1 phase. PMA-induced monocyte-macrophage differentiation also causes a rapid reduction in bcl-2 and strong up-regulation of the adhesion molecule CD11b which is a component of the complement C3 receptor, and also has binding domains for heparin, fibrinogen and ICAM-1 (Delia et.al.1992). CD11b expression is up-regulated on HL-60 with PMA treatment (90% positive cells at 2 days), DMSO on its own however only gave 16% positive cells. All *trans* retinoic acid (ATRA) is used therapeutically in patients with APML (M3) which differentiates the leukaemic clone and increases sensitivity of HL-60 cells to cytosine arabinoside. This was associated with down-regulation of bcl-2 protein expression. K562 erythroleukaemia cells similarly treated with 1μM ATRA for 24 hours also down regulate bcl-2 but without any evidence of apoptosis induction (Fenaux et.al.1996). The half-life of bcl-2 protein in control cells was 20 hours, in the presence of ATRA it fell to 12 hours and was increased in the presence of hydrocortisone to 43 hours. Hydrocortisone has a protective effect on cytosine arabinoside and daunorubicin-treated cells but the mechanism is unclear (Smets et.al.1994). Cytosine arabinoside and daunorubicin are more effective if the bcl-2 level in blasts is first down regulated by ATRA. Blasts are more sensitive to cytosine arabinoside in the presence of G-CSF and less in the presence of GM-CSF. This may be due to the up-regulation of bcl-2 by GM-CSF but an increase in cell cycling induced by G-CSF. Bcl-2 expression in blasts was greater in the presence of GM-CSF or G-CSF plus GM-CSF than G-CSF alone, ATRA however down regulated bcl-2 (Hu et.al.1996).

Because of widespread interest in its efficacy in haematological and other malignancies an antisense to bcl-2 messenger RNA was used to achieve direct therapeutic manipulation of bcl-2 protein levels in AML blast cells in this thesis. This was with a view to either inducing blast cell differentiation or increasing cell sensitivity to standard chemotherapeutic agents independent of their mechanism of action. Induction of apoptosis is not limited to highly proliferative cells (Hickman et.al.1994). It was felt that should bcl-2 be sufficiently down-regulated then the resistance of blast cells to therapy might be tipped towards cell death rather than survival caused by elevated bcl-2 levels.

Antisense uptake in normal peripheral blood cells

In order to achieve baseline values and characterise the binding and uptake kinetics of antisense molecules into haematopoietic cells normal peripheral blood was used as a control system. Antisense phosphodiester (native DNA backbone) and phosphorothioate (where one of the acid phosphate group oxygens is replaced by sulphur) molecules were synthesised with and without a 5'-FAM (fluorescein addition monomer) or 5'-biotin detected by labelled streptavidin. The labels were used to monitor the concentration and kinetics of uptake by flow cytometry and to monitor cellular compartmentalisation of antisense by UV microscopy.

Calibration of antisense uptake

In order to quantify the fluorescence signal associated with cells a calibration system was established. This utilised polycarboxylate spheres with a reactive carboxylate group. These beads were coupled to an 5'-amine labelled cDNA molecule complementary to antisense sequences using EDC coupling reagent (Peirce, UK) according to the manufacturers protocol. Saturating amounts of antisense species were hybridised to the complementary sequence (Biotinylated and FAM-conjugated antisense molecules).

The fluorescent signal associated with saturating amounts of FAM-labelled antisense or biotinylated antisense (detected by streptavidin-PE) hybridised to the complemetary sequence (conjugated to beads) was determined by flow cytometry. Therefore the fluorescence intensity of both the FAM and biotinylated antisense molecules could be directly related to each other through hybridisation to an equivalent amount of cDNA. The FAM-labelled probe was to be used to measure total cellular association (intra- and extracellular) with antisense DNA and the biotinylated probe used to detect only the extracellular component of the antisense association. This could be achieved because the detecting molecule streptavidin is impermeable to cell membranes therefore only the

accessible extracellular component could be detected. Thus the difference in DNA content (total FAM-extracellular Biotin) should relate to the intracellular concentration of antisense uptake.

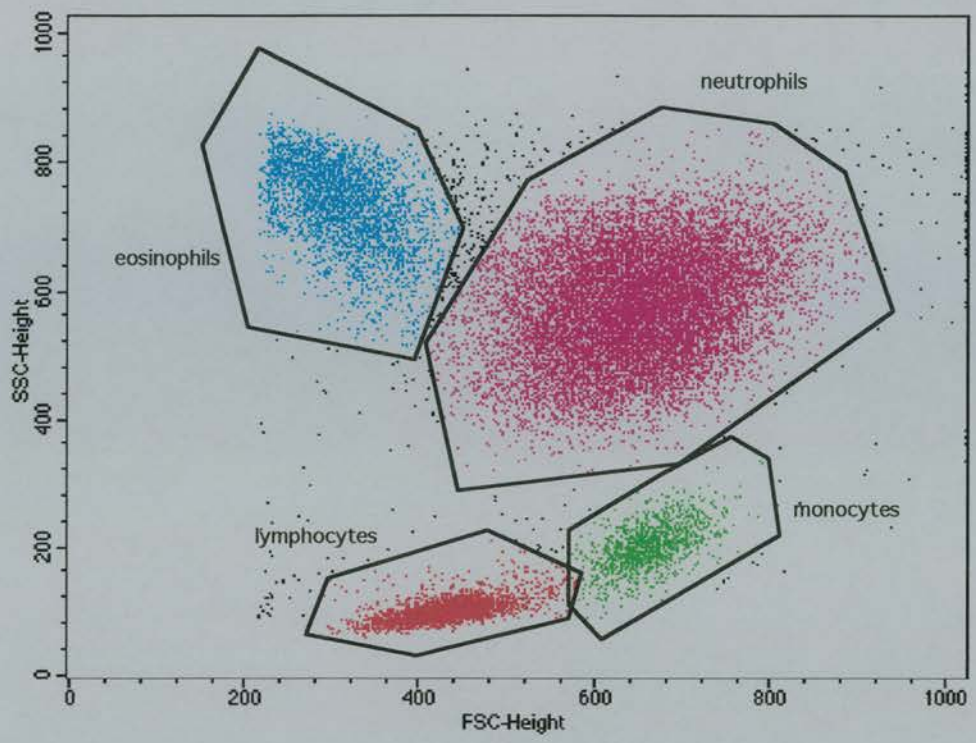
Hybridisation efficiencies between all antisense species to the label used were measured on aliquots of each labelled species which were denatured at 95°C for 5 minutes to dissociate beads from DNA before being spun to remove the spheres. The concentration of oligonucleotide in the supernatant was then measured. The mean concentration of oligonucleotide (± standard deviation) in each supernatant was found to be 30.5 ± 0.8 for FAM-, and 29.8 ± 1.1ng per 10^3 spheres for biotin-labelled antisense respectively. Therefore, it was concluded that there was no difference in hybridisation efficiency. This calibration allowed the determination of correction factors which could then be applied to the fluorescence values to calculate antisense concentrations. For FAM-labelled antisense, the equivalent amount of streptavidin-labelled biotinylated antisense in PE (Fl-2) units was given by the green fluorescence (FL-1) value multiplied by 4.61 and the equivalent of biotinylated antisense in FAM (FL-1) units was given by the red fluorescence (FL-2) value multiplied by 0.216.

Cellular uptake

Having determined calibration settings for antisense values, cells of normal peripheral blood and KG1-a (acute myeloid leukaemia cell line) were studied in a dose and time dependent fashion to determine baseline values for normal and leukaemic cell types. Whole blood, from which red cells had been lysed, were studied in triplicate to determine uptake in granulocytic cell types (neutrophils and eosinophils), lymphocytes and monocytes. Lymphocytes, density-gradient purified followed by adherent and phagocytic-cell depleted, and continuously growing AML cell cultures were also studied.

Naked antisense DNA was incubated with cells for 0, 30, 60, 120 or 240 minutes in the presence of 0, 0.1, 1.0, 10μM antisense-FAM or antisense-biotin in serum free-medium (QBSF-51 Sigma, UK). Cells were washed in PBS, if neccessary surface-labelled with PE-labelled CD19 (B cells); CD3, CD4 or CD8 (T cells) washed again and 40μl propidium iodide solution added to assess cellular viability and cells analysed by flow cytometry. Cells were identified as follows, lymphocytes were of small to intermediate size with little granularity, monocytes were larger and more granular, neutrophils were of intermediate size but with a greater granularity than lymphocytes, and finally eosinophils (where present) were small and extremely granular (**Figure 13**). Lymphocytes were further classified into T and B cells on the basis of surface immunophenotype as described above.

The levels, and kinetics of antisense uptake varied considerably between normal peripheral blood cell types with granulocytic and monocytic cells having far greater antisense association than lymphocytic cells. Monocytes took up most antisense, neutrophils intermediate levels, and lymphocytes the least. Monocytes and neutrophils both showed high levels of extracellular binding at the earliest time point, which then decreased over time in culture, while their intracellular levels steadily increased. Lymphocytes showed much lower levels of cell surface binding, which remained constant throughout the culture period with intracellular concentrations for all but the lowest dose used, had reached maximum levels by 1 hour in culture, KG1-a cells retained lowest levels of antisense association at all time points and doses (**Figs. 14, 15, 16, 17**). Within the lymphocyte population CD19+ B cells were found to have the highest levels of antisense association. There were no differences found in the levels of antisense uptake between CD4 and CD8 positive T cells (**Figure 18a**). KG1-a cells and normal and leukaemic CD34+ cells had levels similar to lymphocytes which were an order of magnitude less than neutrophils and monocytes.



(**Figure 13**). Bi-variate flow-cytometric dot-plot demonstrating the distinctive light scatter characteristics of normal peripheral-blood white cells.

Extracellular association of antisense with cells

In all cell populations extracellular uptake was found to be dose-dependent and non-saturating within the range of doses (0.1 to 10μM) studied. The highest levels of extracellular association were detected at earlier time points (<1hr), with decreases observed over time.

Cellular uptake of antisense into cells

In all cell populations intracellular uptake of antisense was dose and time-dependent, and was not saturated at concentrations of antisense up to 10μM for 4 hours. By 4 hours intracellular concentrations of antisense had reached levels equivalent to the extracellular concentrations achieved within the first hour.

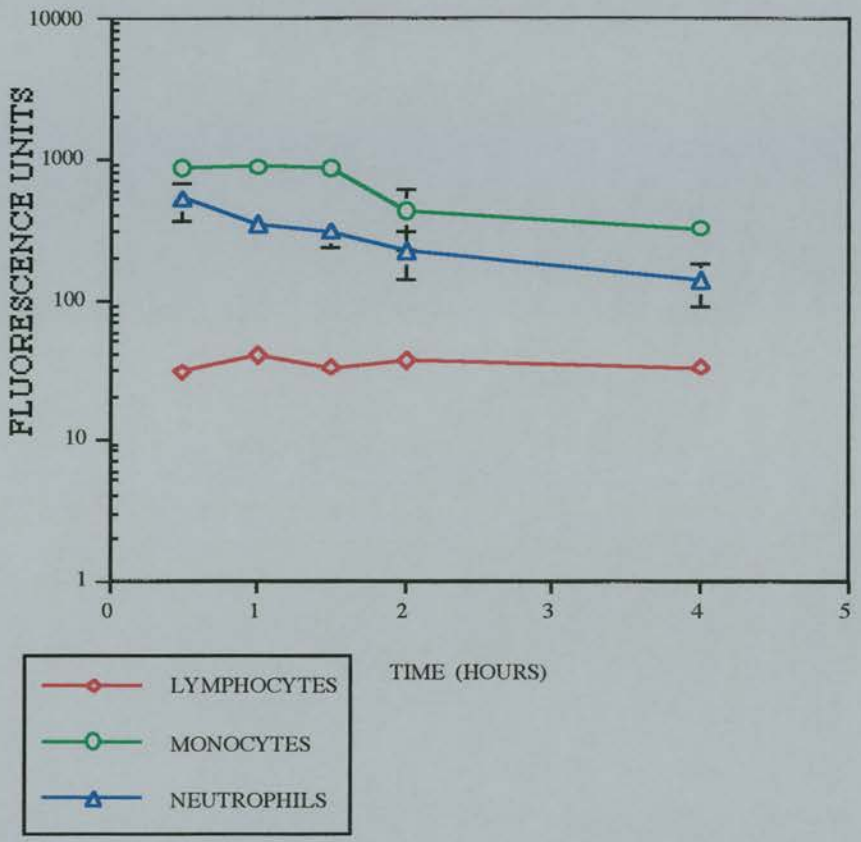
Lymphocytes

At 0.1μM there was no apparent elevation of extracellular antisense levels during the 4 hour incubation period. Similarly very little change in the intracellular levels was seen until 2 hours after addition of the drug. The intra and extracellular levels were similar at this time point however by 4 hours there was a three-fold increase of intracellular over extracelluar levels indicating a possible active uptake at this time point (**Figures 14a, 16a, 16d**).

With 1.0μM drug extracelluar levels at 30 minutes were similar to those seen with 0.1μM, however these levels had trebled by 60 minutes but dropped by 4 hours incubation. This is not reflected in intracelluar levels which at 30 minutes started with six-fold more antisense in intra over extracellular levels which then dropped to a two-fold elevation by 2 hours but then recovered to a maximum of four times extracelluar levels at 4 hours (**Figures 14b, 16b, 16d**).

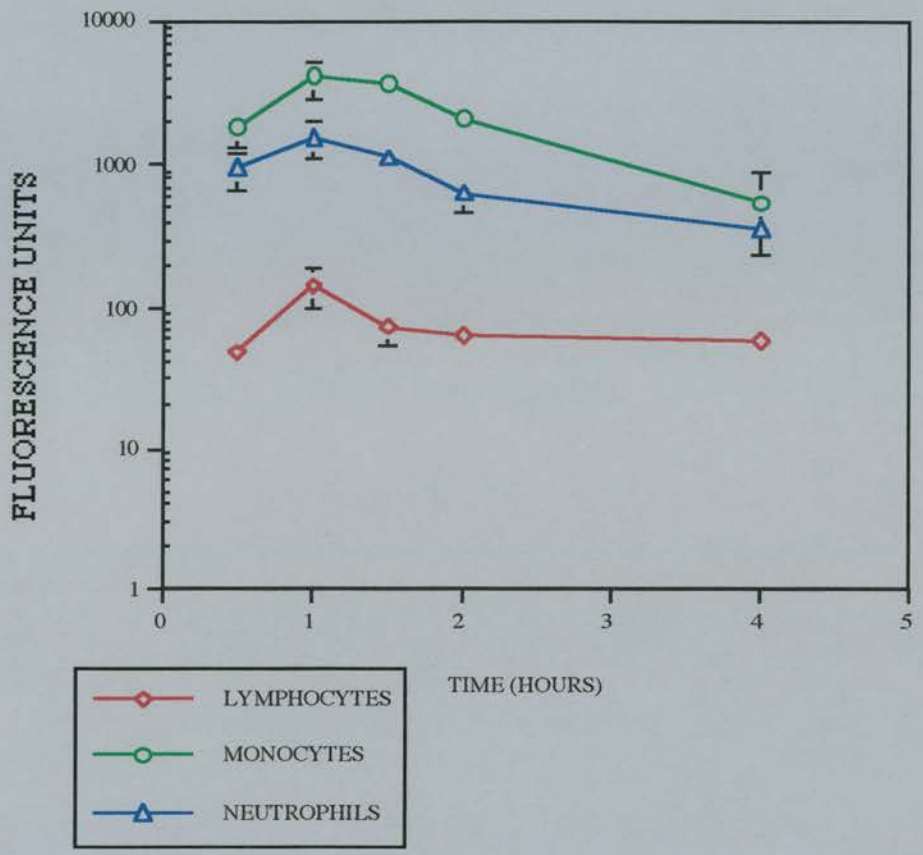
The 10μM antisense dose saw a marked elevation in extracelluar levels (twenty times more than 0.1μM values), a level which remained fairly constant over the 4 hour incubation period. This was not paralleled in intracellular levels which were similar to the 0.1μM values at 30 minutes but rose steadily to plateau at four times the 30 minute concentrations at 4 hours with twice as much inside as outside cells (**Figures 14c, 16c, 16d**).

EXTRACELLULAR BIOTINYLATED ANTISENSE (0.1 μM) ASSOCIATION IN PERIPHERAL BLOOD CELLS



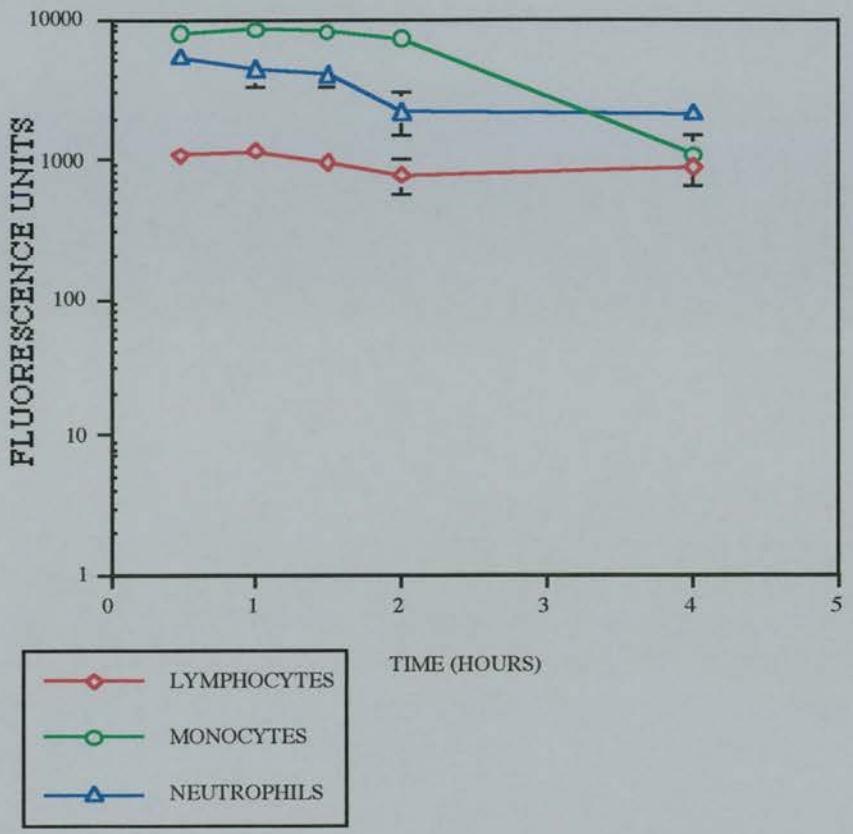
(**Figure 14a**). Showing the extracellular association of a 0.1μM dose of biotinylated antisense in normal peripheral blood cell types.

EXTRACELLULAR BIOTINYLATED ANTISENSE (1.0 μM) ASSOCIATION IN PERIPHERAL BLOOD CELLS

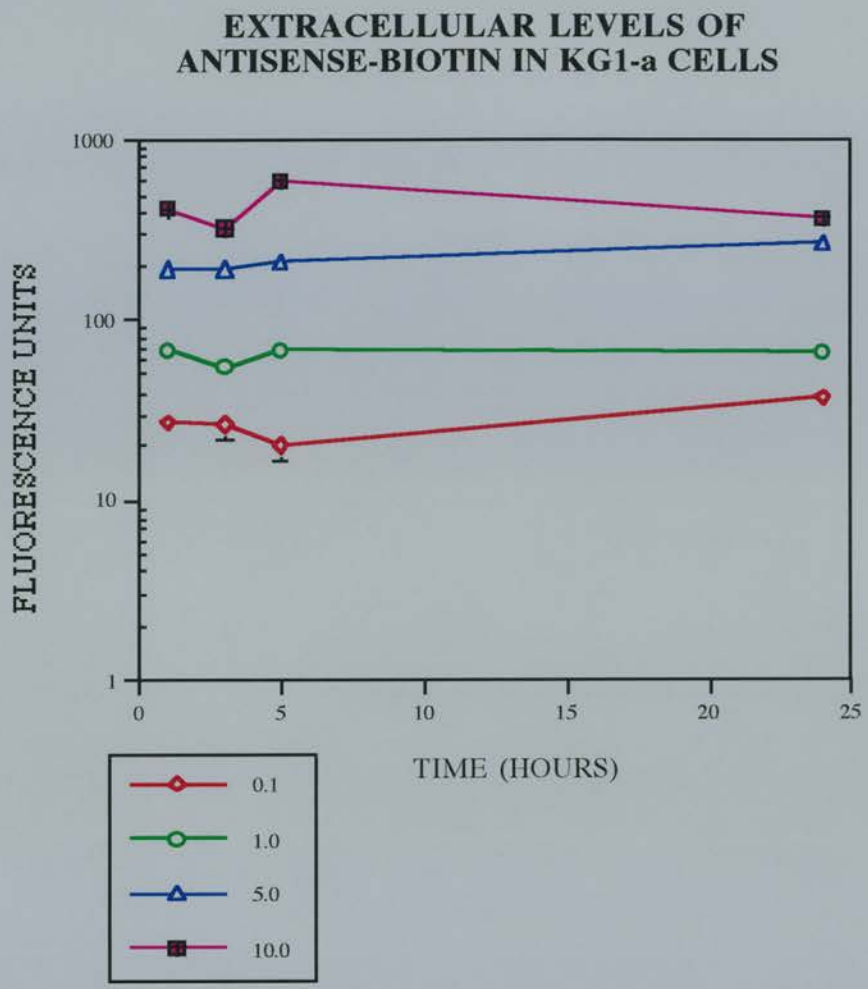


(**Figure 14b**). Showing the extracellular association of a 1.0μM dose of biotinylated antisense in normal peripheral blood cell types.

EXTRACELLULAR BIOTINYLATED ANTISENSE (10 μM) ASSOCIATION IN PERIPHERAL BLOOD CELLS



(**Figure 14c**). Showing the extracellular association of a 10μM dose of biotinylated antisense in normal peripheral blood cell types.



(**Figure 15**). Showing the extracellular association of all doses (0.1-10μM) of biotinylated antisense in KG1-a (AML) cells.

Monocytes

Monocytes showed the greatest levels of extracellular antisense association. At 0.1μM antisense monocytes had a higher association of drug than either lymphocytes (20x values) or neutrophils (1.5x values) within the first half-hour of incubation. Similar levels were found inside as outside the cells indicating equilibrium and suggesting this might be passive diffusion at this time point. Extracellular levels remained constant over the first 90 minutes when the levels dropped by half at 2 hours and to one third 30 minute values by 4 hours. However intracellular levels rose consistently throughout the period of incubation until by 4 hours there was nine times as much inside as outside the cells. Furthermore there was one hundred times more than inside lymphocytes and two times more than inside neutrophils at the same time point (**Figures 14a, 16a, 16e**).

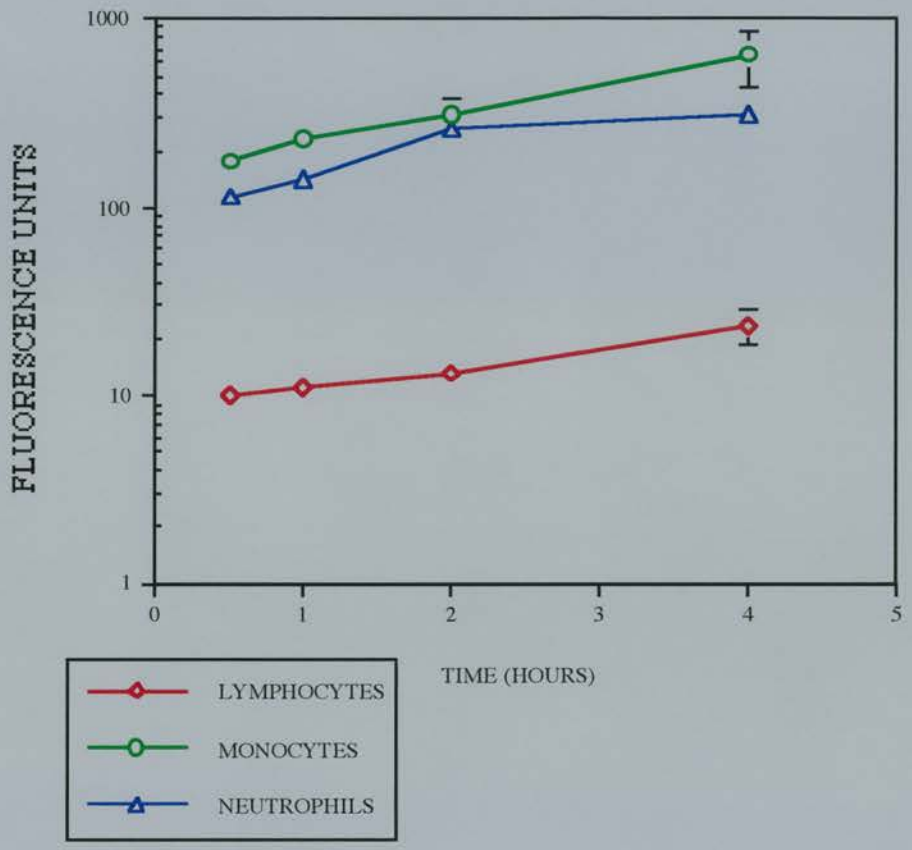
Using 1.0μM drug demonstrated that at 30 minutes there was again an equal amount of antisense inside as outside the cells. Again extracellular levels dropped, such that by 4 hours there was one quarter as much outside cells as at the 30 minute values. There was however a fourteen-fold increase in the amount of drug inside the cells at this time point. Intracellular values interestingly had remained constant until 2 hours when the levels started to incrementally rise (**Figures 14b, 16b, 16e**).

At 10μM antisense there was a three-fold increase in extracellular levels over the 1.0μM values by 30 minutes. This level remained constant over the first 2 hours until 4 hours when levels fell by a factor of seven over the 30 minute values. Intracellular values showed a doubling over the first 60 minutes, remained constant over the next 60 minutes and doubled again by 4 hours. By this time there was twelve times as much antisense inside the cells as outside (similar values to 0.1 and 1.0μM). Intracellular values at this time point were tenfold more than lymphocytes and two times more than neutrophils at the same drug concentration (**Figures 14c, 16c, 16e**).

Neutrophils

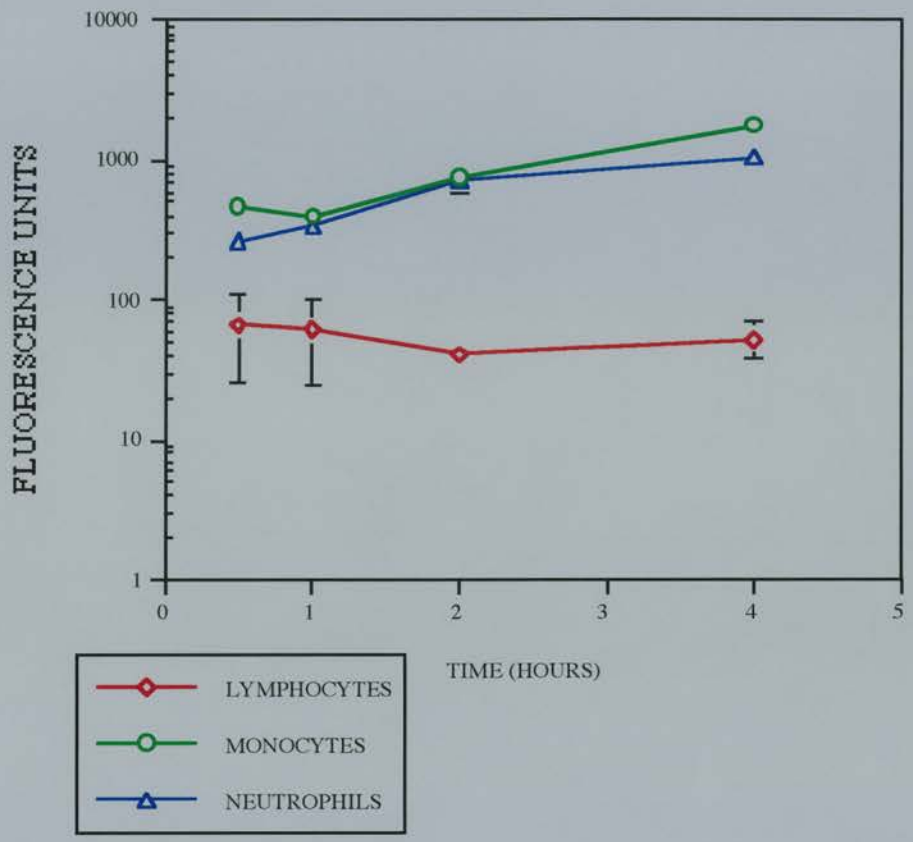
With 0.1μM drug by 30 minutes incubation antisense levels in neutrophils were approximately twenty-fold more than in lymphocytes with equal amounts of drug found inside neutrophils as associated with membranes. However with increasing time there was an incremental decrease in extracellular levels of antisense to levels one quarter the thirty minute values. However intracellular values had doubled in the first 2 hours at which time levels plateaued at two times the extracellular levels and ten-fold more than lymphocytes and half the monocytic level (**Figures 14a, 16a, 16f**).

CELLULAR UPTAKE OF FAM-LABELLED ANTISENSE (0.1 μM) IN PERIPHERAL BLOOD CELLS



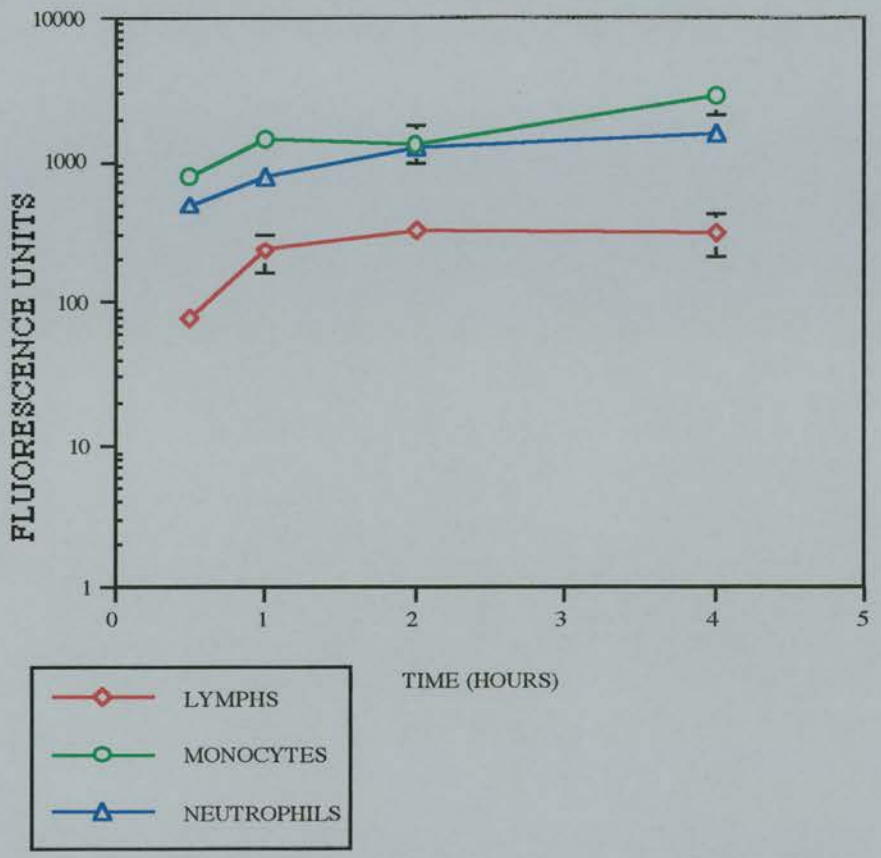
(**Figure 16a**). The intracellular compartmentalisation of a 0.1μM dose of FAM-labelled antisense in normal peripheral blood cell types.

CELLULAR UPTAKE OF FAM-LABELLED ANTISENSE (1.0 μM) IN PERIPHERAL BLOOD CELLS



(**Figure 16b**). The intracellular compartmentalisation of a 1.0μM dose of FAM-labelled antisense in normal peripheral blood cell types.

CELLULAR UPTAKE OF FAM-LABELLED ANTISENSE (10 μM) IN PERIPHERAL BLOOD CELLS



(**Figure 16c**). The intracellular compartmentalisation of a 10μM dose of FAM-labelled antisense in normal peripheral blood cell types.

At thirty minutes after addition of 1.0μM antisense extracellular levels were double that of 0.1μM antisense values. These levels had effectively doubled by 60 minutes but rapidly decreased such that 4 hour values were one quarter of the 90 minute levels. Intracelluar levels meanwhile remained constant over the first 60 minutes and were similar to extracellular levels. However by 2 hours five times as much signal was associated inside as outside the cell the levels of which had increased to fourteen times the extracelluar values at 4 hours (**Figures 14b, 16b, 16f**).

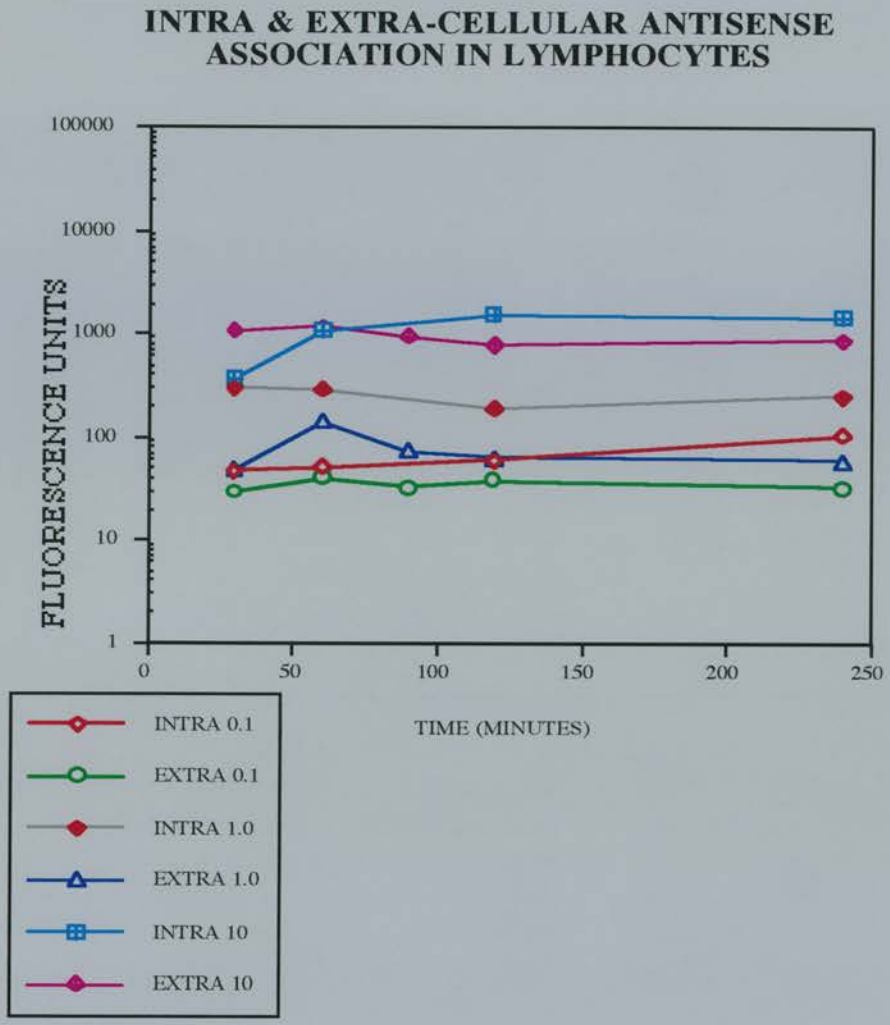
10μM antisense gave extracellular 30 minute levels which were two times those of intracellular compartments. Levels remained consistent until 90 minutes incubation but dropped by 50% by 2 hours, and then remained constant until the end of the study at 4 hours. Intracellular levels rose incrementally over the four hour period and were similar to extracellular levels until 2 hours when a two-fold increase was seen, which then rose to three times extracelluar levels by four hours. At this time point levels were half of monocyte values and five times those of lymphocytes (**Figures 14c, 16c, 16f**).

KG1-a (AML) cells

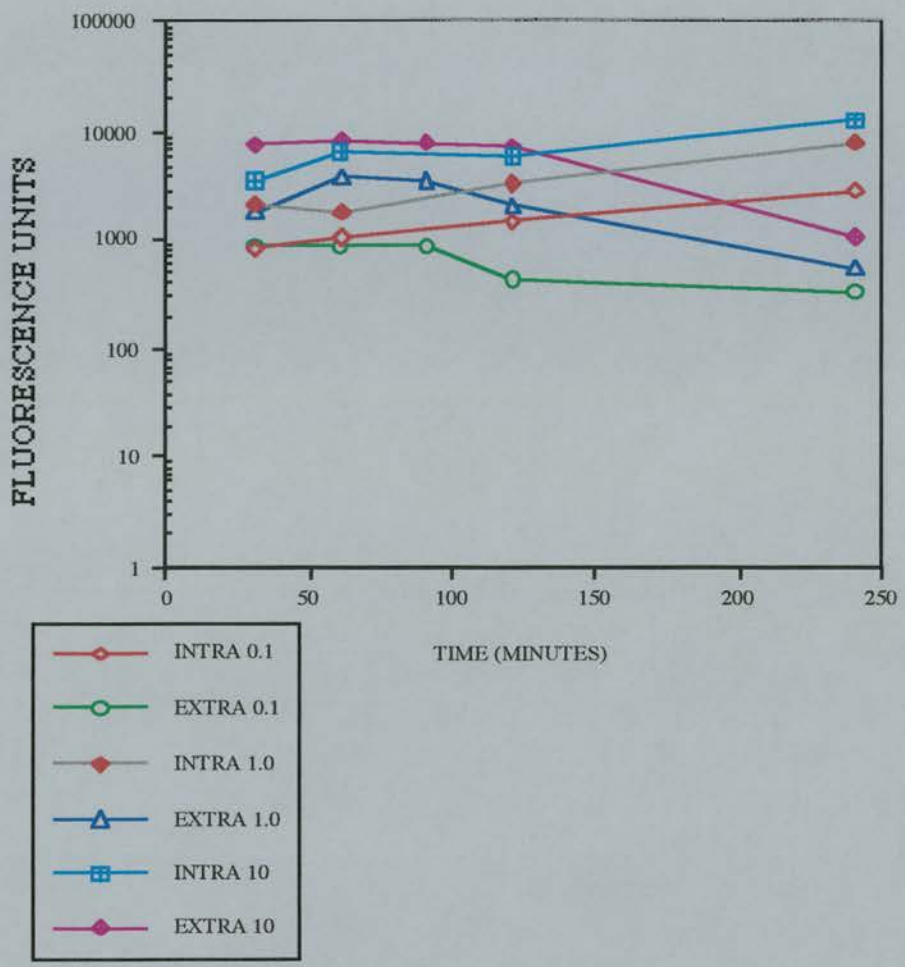
KG1-a cells treated with 0.1μM antisense had extra and intracellular levels of drug similar to lymphocytes at 30 minutes. There was no change in the extracellular level which remained low over the 5 hour incubation. Intracellular levels remained consistent until 3 hours when there was approximately two times as much inside as outside cells with levels again similar to lymphocytes (**Figures 15, 16g, 17**).

1.0μM antisense treated cells had extracellular levels more than two times greater than 0.1μM antisense levels at 30 minutes. These levels again remained constant during the study. Intracellular levels rose slowly such that by 5 hours there was twice as much inside cells as found at 30 minutes, however intra and extracellular levels were similar indicating that passive diffusion may account for most of the uptake. Again levels were found to be similar to those of lymphocytes (**Figures 15, 16g, 17**).

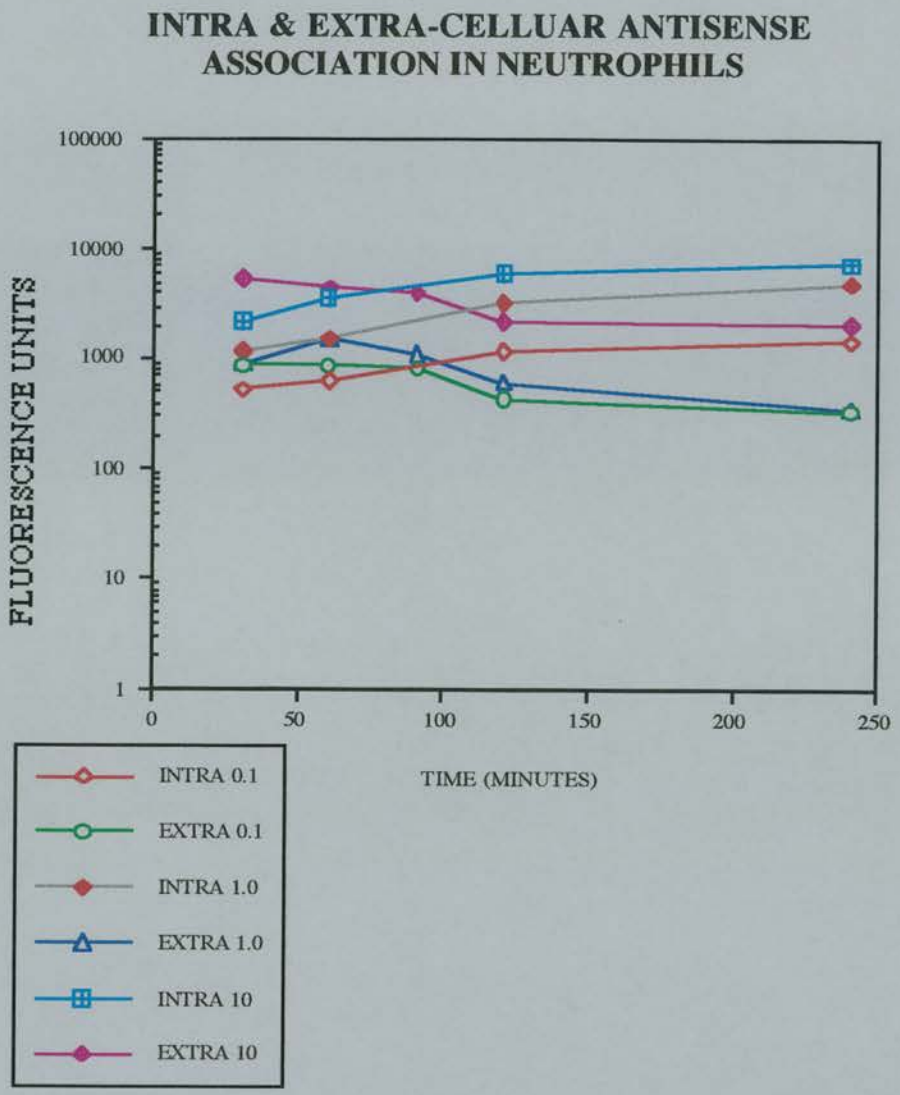
10μM antisense levels on the surface of cells was found to be fifteen times more than that found with 0.1μM drug at 30 minutes. This level remained constant over the 5 hour incubation period, however there was a small decrease found at 30 minutes with an increase found at 5 hours. Intracellular levels remained low however at between one fifth to one half of levels found on the cell surface. These levels were lower even than levels found in lymphocytes (one seventh) with a similar extracellular value. This may represent relatively poor cellular uptake or an efficient system for removal of drug in these cells (**Figures 15, 16g, 17**).



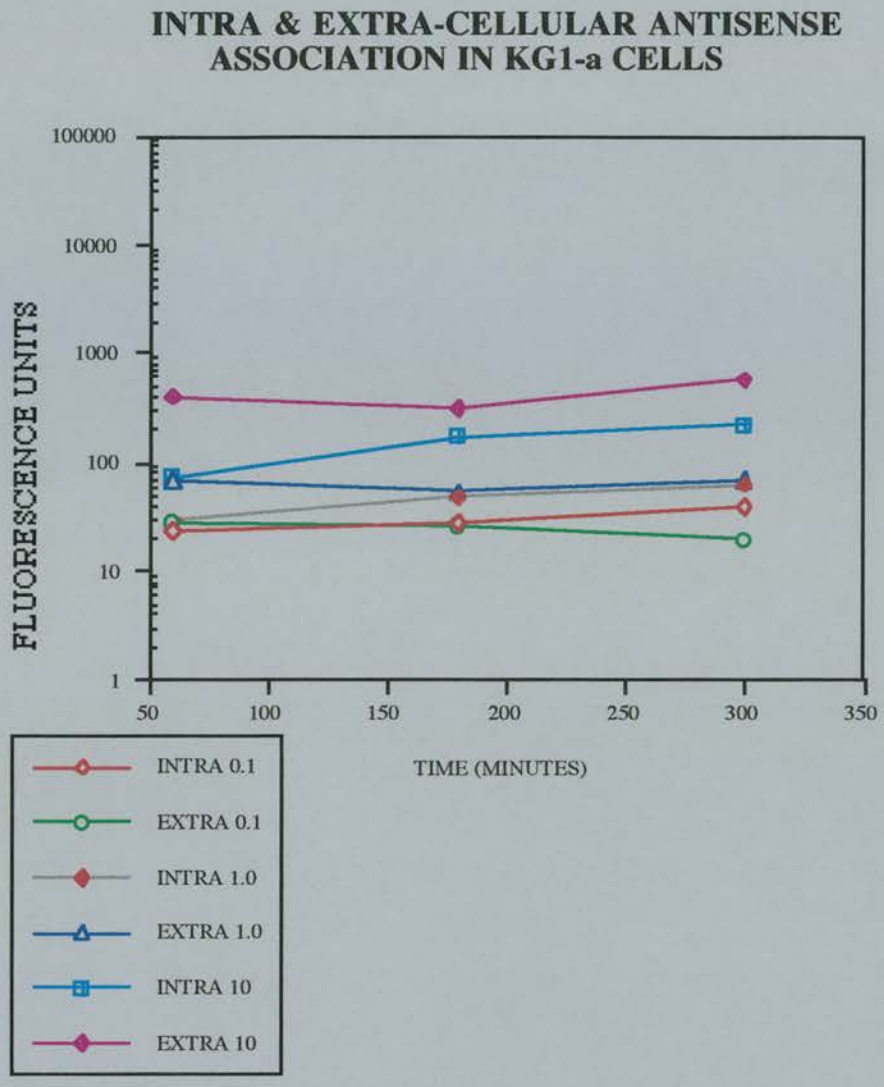
(**Figure 16d**). The intra and extracellular compartmentalisation of antisense in normal lymphocytes.



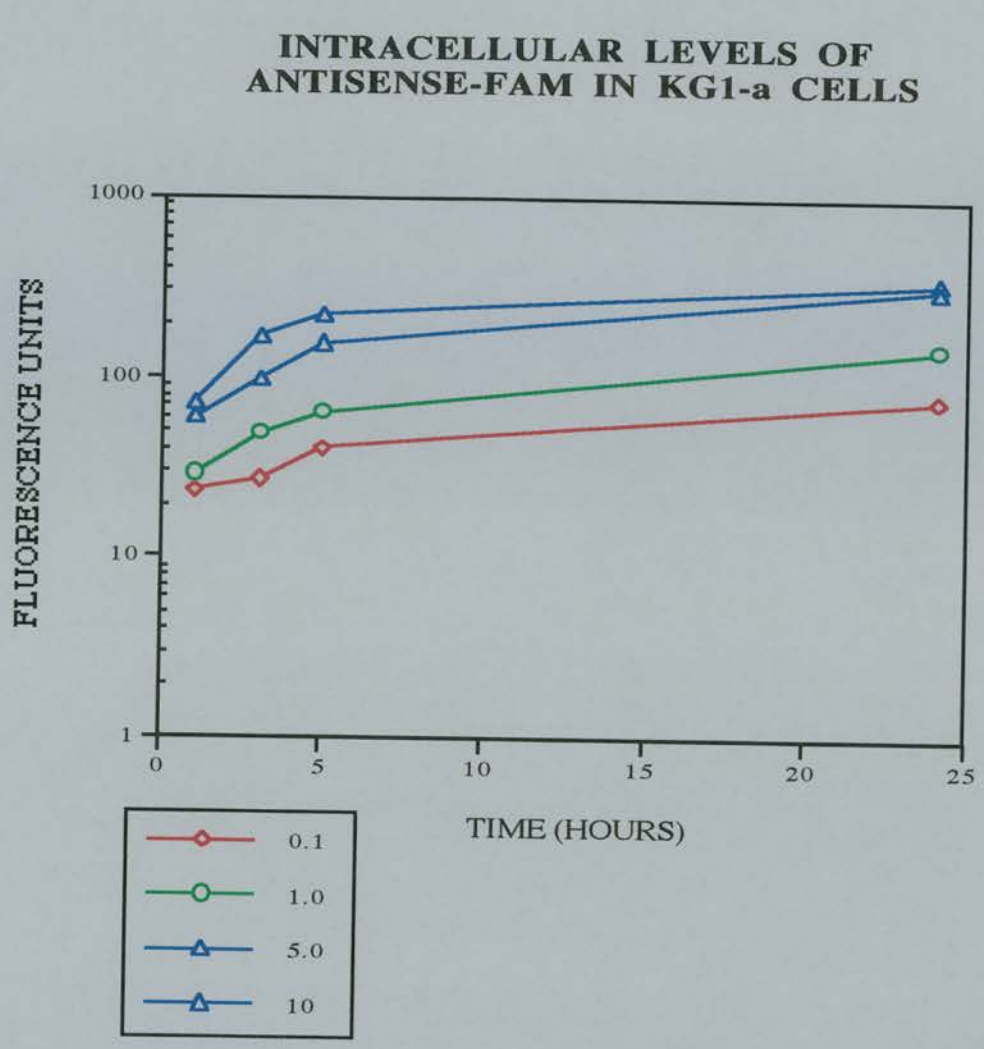
(**Figure 16e**). Intra and extracellular compartmentalisation of antisense in monocytes.



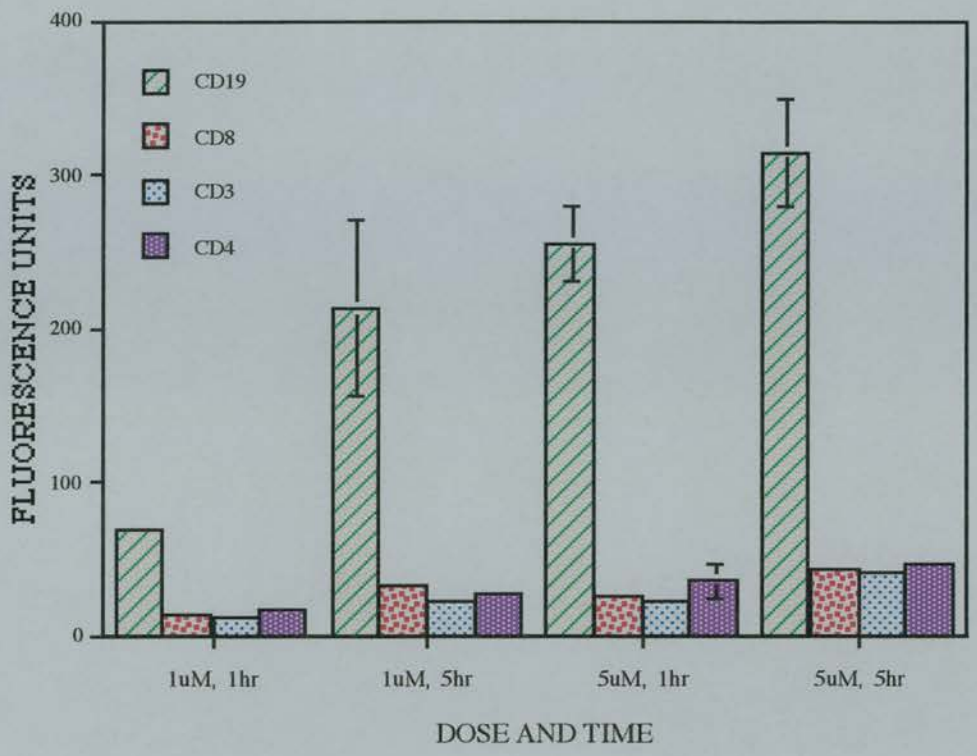
(**Figure 16f**). Intra and extracellular association of antisense in neutrophils.



(**Figure 16g**). The intra and extracellular compartmentalisation of antisense in KG1-a (AML) cells.



(**Figure 17**). The intracellular compartmentalisation of all (0.1-10μM) doses of FAM-labelled antisense in KG1-a (AML) cells.



(**Figure 18**). The intracellular compartmentalisation of a 1 and 5μM dose of FAM-labelled antisense in normal peripheral blood lymphocyte sub-types. CD19+ B cells are seen to take up 10x more than all types of T cells.

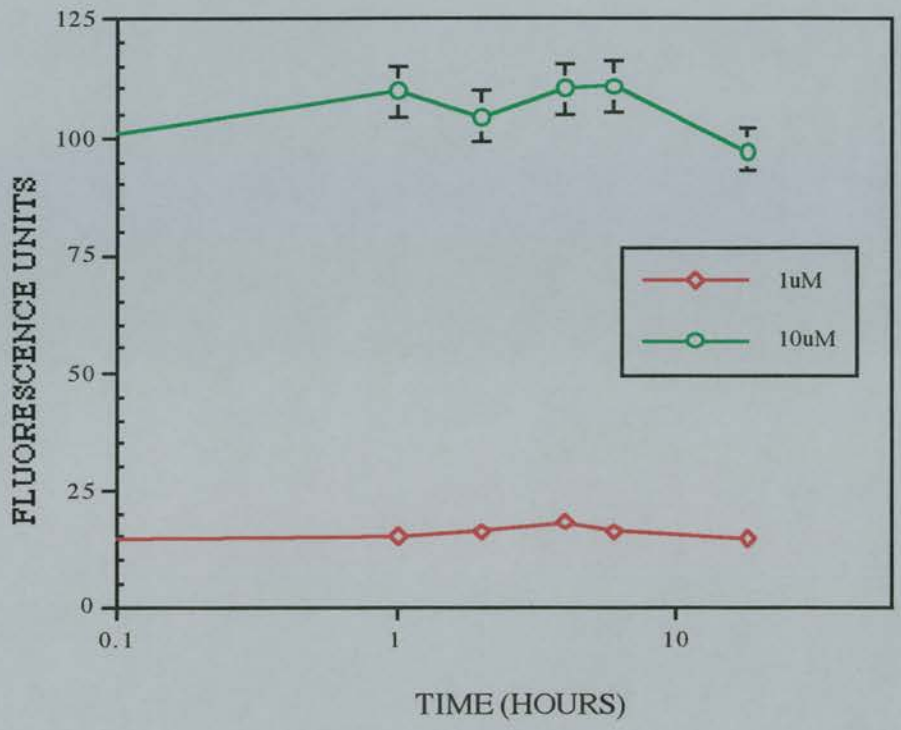
All cells

Antisense association with peripheral blood leucocytes was rapid with a fluorescent signal associated with in monocytes by 30 minutes. There is a dip in the extracellular concentration of oligo in myeloid cells which is not reflected in a change in intracellular concentration. This may relate to receptor-ligand internalisation or down-regulation. Levels of antisense uptake are greatest in myeloid cells with the following ranking: monocytes > neutrophils > B cells > T cells (CD4 and CD8 cells equivalent) (**Figure 18**).

Efflux of antisense

Once inside lymphocytes, antisense exited very slowly. No significant efflux was detected up to 18 hours after antisense removal from culture. In light of the long-term stability of antisense within cells, it seems clear that a bolus, or short pulse, administration of the oligonucleotide should be capable of achieving effective intracellular concentrations without many of the concomitant side effects (**Figure 19**).

ANTISENSE EFFLUX FROM LYMPHOCYTES



(**Figure 19**). Antisense efflux from lymphocytes was negligible over an 18 hour period following loading with either 1.0 or 10μM antisense-FAM.

Antisense quenching

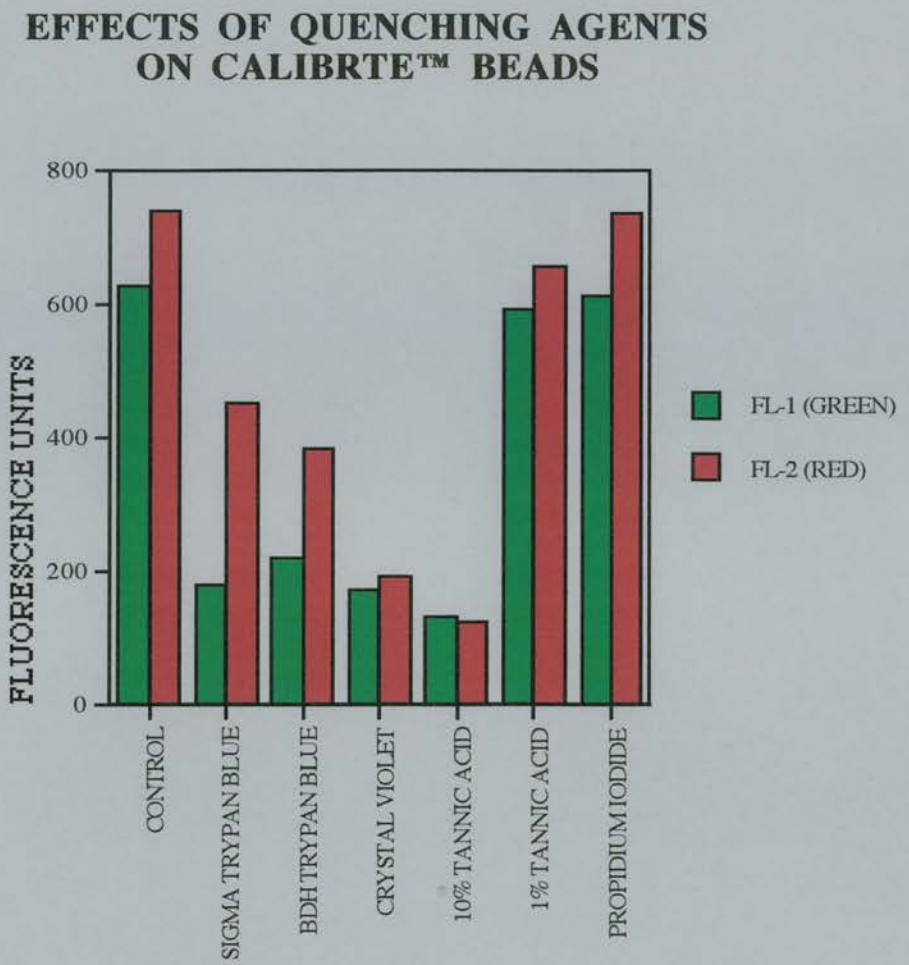
As described above the initial study aim was to use FAM-labelled antisense to quantify total antisense association and biotinylated oligo (detected by streptavidin) to measure the extracellular component. However the arithmetic did not seem to justify that this assumption could be correct. There was an anomaly in the amounts of DNA detected and frequently the biotin signal was equivalent to or greater than that from FAM. Thus it appeared that something was in fact quenching the signal from this dye. An investigation of fluorescence quenching agents was therefore sought. The effects of known quenching agents on fluorescent signals was monitored for similarity to that seen with the FAM label.

Trypan blue, crystal violet and tannic acid are agents known to effectively quench fluorescence signals by absorbing the emitted fluorescence light. 0.05% trypan blue was shown to quench the stable membrane dye PKH-26 but not intracellular ethidium bromide, tannic acid was shown to quench FITC-labelled yeast (Giaimis et.al.1994; van Amersfoot et.al.1994). This results from a phenomenon known as fluorescence resonance energy transfer where the emission wavelength of the first dye is absorbed as the excitation wavelength of a second dye which results in a quenching of fluorescence. Therefore the effects of these agents on green and red fluorescence from beads (Calibrite™ Becton Dickinson, Oxford, UK) or viable cells stained with monoclonal antibody was determined. Trypan blue (2mg/ml) from two sources (BDH and Sigma, UK) was studied as they had previously been described as possessing differing quenching abilities, crystal violet (2mg/ml) (BDH, UK) or Tannic acid at 1 and 10 % were added to 50µl Calibrite™ Beads and incubated for 5 minutes at room temperature and the beads' residual fluorescence measured by flow cytometry . There was a marked and obvious quenching of both the Fl-1 and Fl-2 signals when the beads were quenched with both forms of the trypan blue with Sigma Trypan blue being slightly more effective in FL1 and BDH Trypan blue on Fl-2. However overall the green fluorescence was more effectively quenched than the red. Crystal violet and 1% tannic acid were able to effectively quench the signal in both FL-1 and 2, however 10% tannic acid caused severe flocculation of the beads without any quenching. The action of propidium iodide (40µl of a 0.025% solution) was similarly measured in the assay since this and the streptavidin-PE were the only differences between the biotin and FAM labelled cells. However PI had no effect on the fluorescence of the beads (**Figure 20**). Similarly the effects of these agents on monoclonal antibody stained peripheral blood mononuclear cells revealed a similar pattern. Cells were stained with either CD38-FITC, CD45-FITC, CD45-PE or CD3-PE and the fluorescence measured after 5 minutes at room temperature. Again quenching with trypan blue was seen though not as dramatic as with the calibration beads nor did the effectiveness appear to

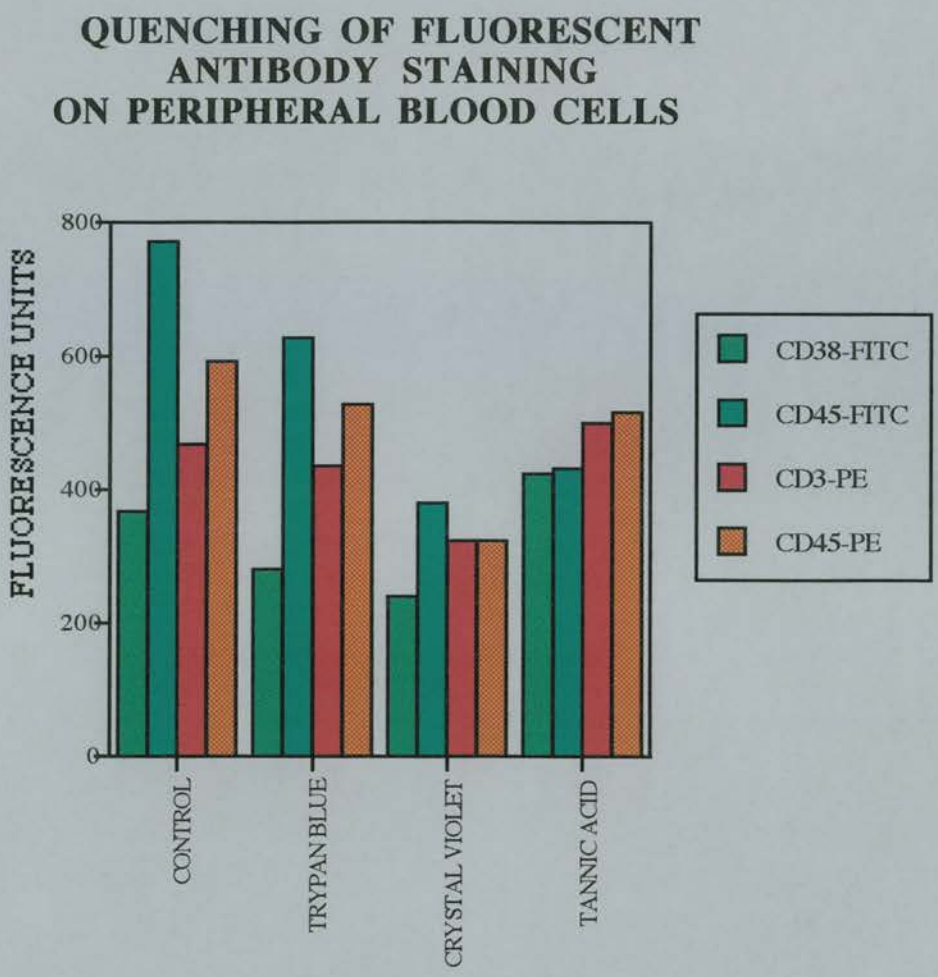
be constant between different antibodies with differing binding sites. This may result from the differences in the initial intensity of staining and may also be related to the F:P ratio with more heavily labelled probes being more effectively quenched due to the proximity of flourophores eg. CD45-FITC versus CD45-PE (**Figure 21**). Crystal violet and tannic acid at both concentrations were found to destroy normal cellular morphology and so were rejected as unsuitable for further use.

An attempt therefore was made to look at the potential for quenching agents on the fluorescent signal both on FAM-labelled DNA-conjugated beads or on leukaemic cells treated with 5μM antisense for 24 hours. This time trypan blue was similarly found to be an effective agent at quenching the fluorescence signal though the effect was minimal at this time point.

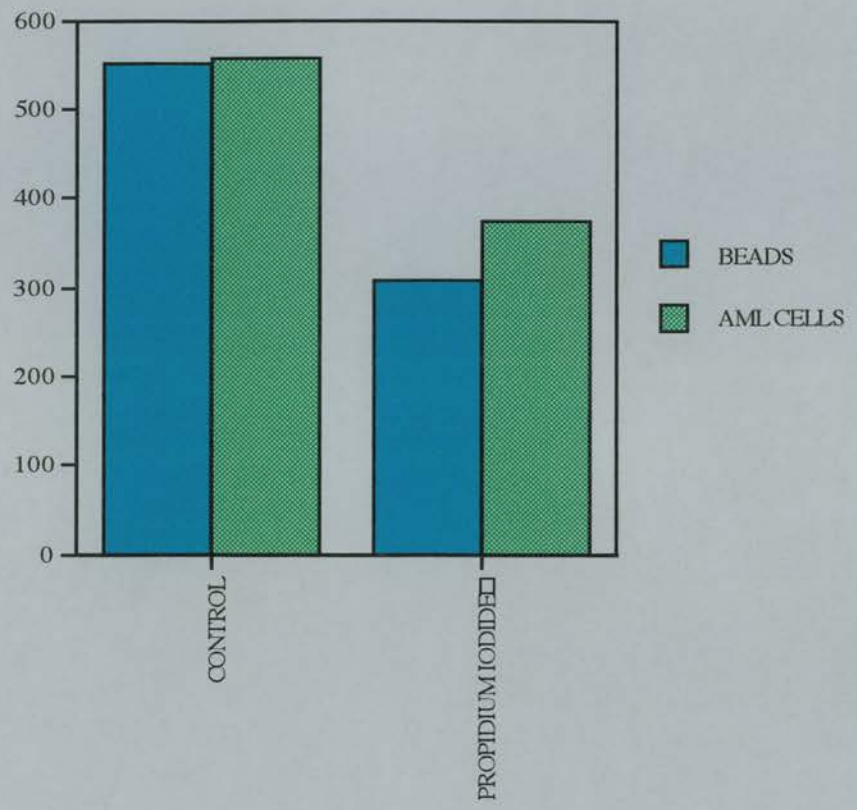
Similarly PI was monitored for its effectiveness since a previous study using ethidium bromide (Fattorossi et.al.1989; Vogel et.al.1994) had been shown to effectively quench the FITC signal from labelled *Candida albicans.* There was an obvious marked reduction in the fluorescent signal from both beads and cells which was wholly surprising given the lack of quenching seen with antibody-labelled beads or cells (**Figure 22**). When monitored by fluorimetry the data was confirmed, both trypan blue and propidium iodide were able to quench the signal from FAM labelled antisense DNA. Thus the initial discrepancy between the FAM and biotin labelled antisense uptake had been revealed. The propidium iodide initially used as a viability stain had effectively quenched the extracellular fluorescence revealing only the intracellular compartment to be visualised both by flow cytometry and fluorescent microscopy. Though this appeared to be a time-dependent phenomenon it merely reflected changes in the cellular partitioning of antisense with time such that with increasing time there was more intracellular fluorescence and therefore the portion quenched by PI became reduced.



(**Figure 20**). Trypan blue, crystal violet and 10% tannic acid are all effective at quenching fluorescent beads, propidium iodide however has no effect.



(**Figure 21**). The effects of quenching agents on antibody staining. Trypan blue has a variable effect, crystal violet and tannic acid though effective destroy cell morphology.



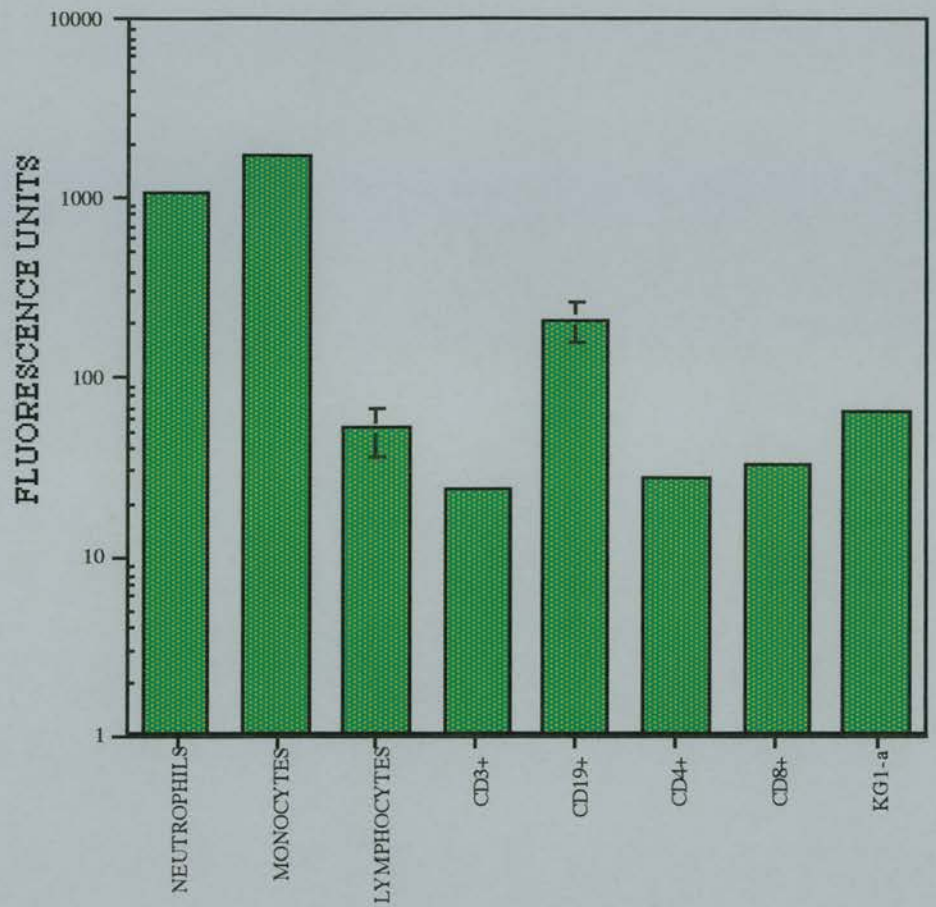
(**Figure 22**). Propidium iodide is able to quench the signal from FAM-labelled antisense linked to beads or cells. Beads were quenched to their background levels whereas cells had residual signal from intracellular antisense.

It was therefore possible to compare the intracellular concentration of a single dose (1μM at 4 hours) of antisense in differing cell types to obtain a direct comparison of uptake. Monocytes were found to have the highest levels with neutrophils taking up similar but slightly lower levels. CD19 positive B cells and CD34 positive AML patient blasts took up similar levels of antisense with similar to but slightly greater than normal CD34 positive cells. T cells took up least amounts of antisense with no apparent differences between CD3, 4, and 8 positive cells (**Figure 23**).

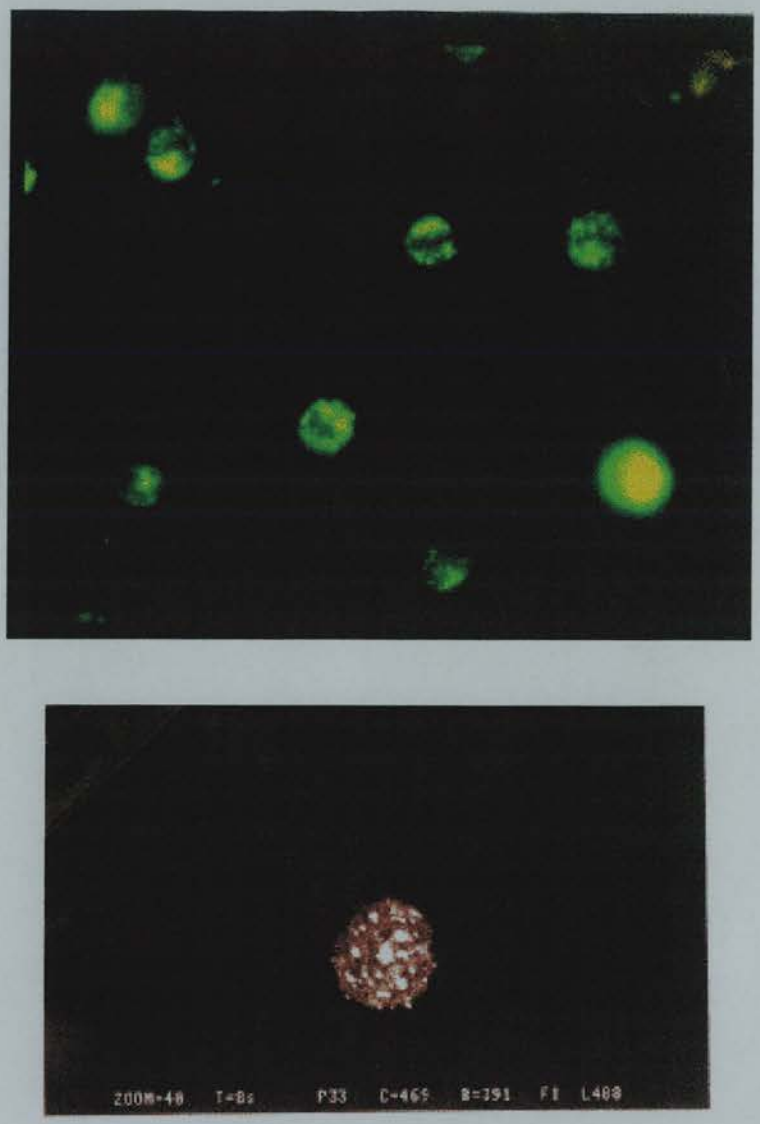
Enhancers of antisense uptake

Having now been satisfied that there was antisense uptake into these cells confocal microscopy revealed that the majority of antisense appeared to be sequestered in cytoplasmic vesicles (**Figure 24**) which is in agreement with other authors findings (Bergan et.al.1996). In control cells treated with antisense the oligo is sequestered to the cytoplasmic vesicles. However following electroporation the oligo localised directly to the nucleus and non-vesicular cytoplasmic structures within the cytoplasm. No effect of c-myc oligos were seen without electroporation. This method allowed up to 7 times more signal inside cells following electroporation than those incubated with naked DNA (Bergan et.al.1996).

INTRACELLULAR LEVELS OF 1.0μM ANTISENSE AT 4 HOURS

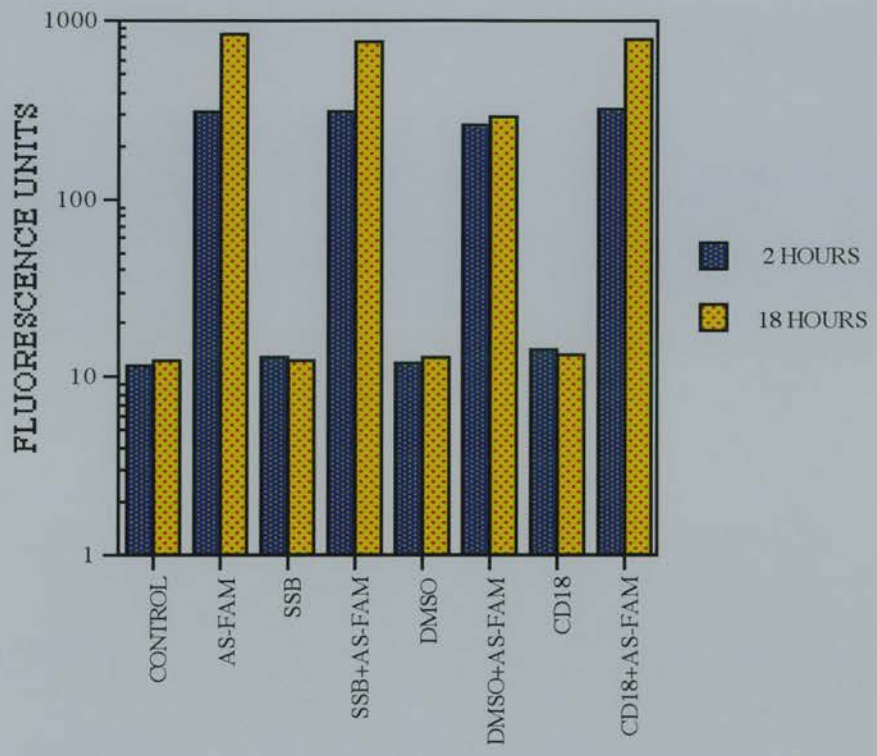


(**Figure 23**). A single dose (1.0μM) of antisense for 4 hours reveals a decreasing magnitude of antisense uptake monocytes>neutrophils>B cells>T cells = KG1-a cells.



(Figure 24). Top: Fluorescence microscopy and Bottom: confocal laser microcopy images of intra-vesicular compartmentalisation of antisense inside cells.

EFFECTS OF AGENTS ON UPTAKE OF ANTISENSE IN KG1-a CELLS



(**Figure 25**). The effects of agents on antisense uptake into cells. DMSO may improve cytoplasmic delivery and together with SSB may reduce potential secondary structure formation. The CD18 antibody may interfere with integrin/Mac-1-mediated antisense cellular internalisation.

In K562 (an erythroleukaemia cell line) cells an increase in transfection efficiency could be achieved using transferrin-conjugated antisense (Bergan et.al.1996; Cotten et.al.1990). Rapidly dividing cells and cells synthesising haem (neoplastic or haematopoietic) require elevated iron and a 20 hour incubation with desferrioxamine (50μM) was able to increase the transferrin receptor four fold. This related to a 5-15 fold increase in DNA tranfection when performed in the presence of 100μM chloroquine (4 hours) (Schultz et.al.1997). The antisense is sequestered in cytoplasmic vesicles and chloroquine acts to increase lysosomal pH, causing them to rupture and release contents into the cytoplasm thus improving the bioavalibility of the drug. However this type of method is costly, cumbersome and time consuming so a simpler more direct approach was sought.

DMSO (10%), an agent commonly used for bacterial transformation of DNA, was co-incubated with antisense molecules to see whether transfection efficiency could be improved. Similarly sterile ammonium chloride (100μM) was also used to see if this weak base could be used to increase lysosomal pH and thus cause rupture of vacuoles and release antisense into the cytoplasm. However in both these approaches no increase in antisense was seen but an increase in cytotoxicity was apparent (**Figure 25**).

Liposomal transfection using Lipofectin™ was attempted again to increase the availibility of antisense to cytoplasmic targets. A solution of antisense in serum-free medium was added to a solution of Lipofectin™ in serum-free medium and incubated for 15 minutes at 37°C. Cells were incubated in the presence of the liposomal-antisense complex for 4 hours at 37°C, 5% CO_2. Cells were then washed twice in serum-free medium, surface stained with fluorescent antibody when required, and analysed. However the results again were variable with either poor transfection or high levels of cytotoxicity. Also microscopy revealed poor fusion of the liposomal complex to many of the cells.

Streptolysin O was used as an agent for reversible permeabilisation of cell membranes (Spiller et.al.1995). An optimal dose and time of incubation was sought to deliver antisense molecules directly to cytoplasmic and nuclear targets. In the presence of the flourescent vital stains, propidium iodide and fluorescein di-acetate, non-permeabilised viable cells should be green only; permeabilised non-viable cells red only and permeabilised viable cells should have a red nucleus with green cytoplasm. However using this combination of propidium iodide and fluorescein di-acetate solution it was difficult to adjust the compensation settings on the flow cytometer to allow discrimination of the red and green signals. It also proved to be an unreliable technique in that frequently there would be no permeabilisation at all, then under identical

conditions there would be massive cytotoxicity. These methods were therefore abandoned in favour of the simpler, though less effective, direct incubation system.

Biologic effects of bcl-2 antisense

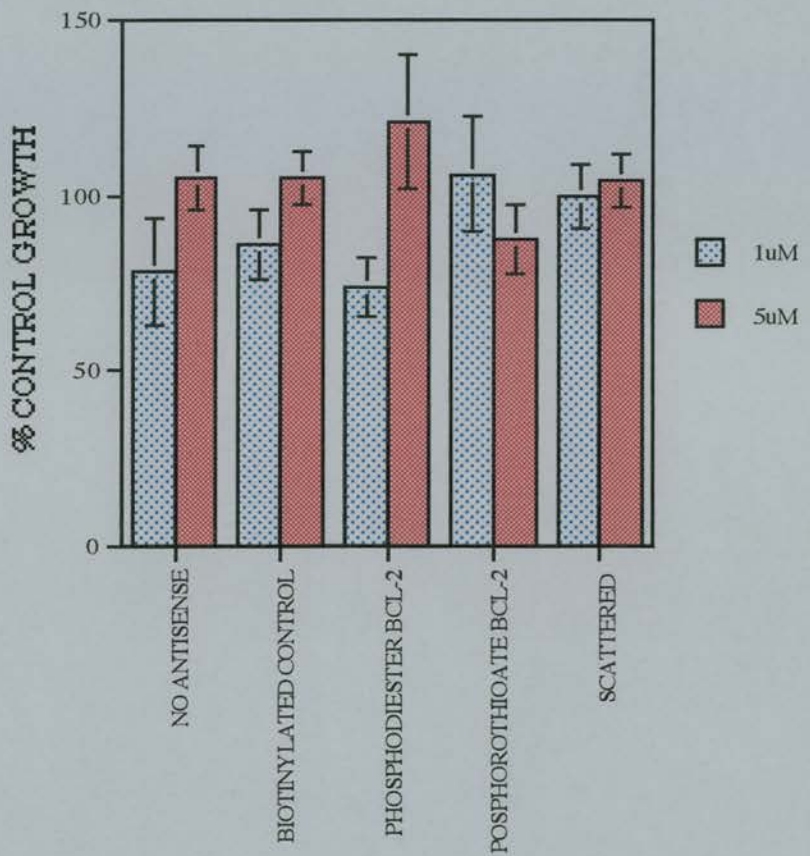
Having established that the bcl-2 antisense did not overlap with any other known protein sequence GenBank search (**Appendix 1**) and that antisense was available to an intracellular compartment its biologic effects on leukaemic colony formation and effects on bcl-2 protein expression were studied. Cells were incubated in the presence of 5μM antisense molecules to look for specific and non-specific effects of antisense molecules. Other authors have described the effective use of antisense in haematologic malignancy with an emphasis on purging of leukaemic cells from autologous bone marrow and leukapheresis products. c-myc (Bergan et.al.1996), Interleukin-1β converting enzyme (Stosic-Grujicic et.al.1995) and bcl-2 (Campos et.al.1994) have all been described as potential therapeutic targets in AML.

Antisense effects on proliferation

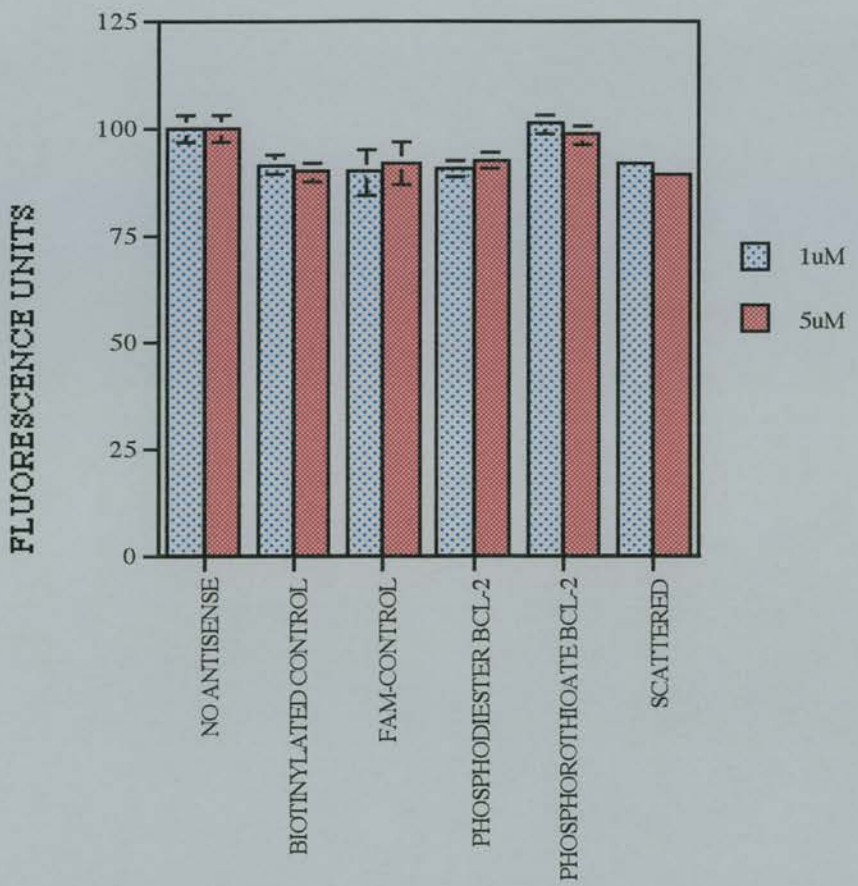
Therefore of the 77 AML samples studied for colony formation those 18 patients who demonstrated any degree of autologous growth were further selected and treated with either 5μM antisense bcl-2, control nonsense antisense or an anti-GM-CSF antibody to look for cytostatic or cytotoxic effects. No patients showed any demonstrable reduction in colony growth with either of the antisense species at day 3 or 7 post-incubation. Of patients treated with an anti GM-CSF antibody 5 out of 18 (28%) patients showed a partial reduction in the number of colonies formed though none showed complete abrogation of growth (**Figure 11d**).

The effects of antisense on the ability of KG1-a cells to form colonies *in vitro* was studied using 10μM antisense. No effects on colony formation were found (**Figure 26**). Similarly the effects of antisense species were monitored for anti-proliferative effects using an MTT dye reduction assay. Briefly 10^5 KG1-a cells were incubated for hours with 1 and 5μM antisense bcl-2 (phosphorothioate and phosphodiester forms), nonsense bcl-2, and control antisense with a limited reduction in cell growth seen with all species excepting the bcl-2 phosphorothioate antisense (**Figure 27**). These antisense effects are most probably non-specific toxic polyanionic effects which have been described by other authors (Stein, 1995).

ANTISENSE EFFECTS ON COLONY FORMATION



(**Figure 26**). Limited effects of antisense were seen on the ability of KG1-a cells to form colonies in methylcellulose.



(**Figure 27**). No effects of antisense were seen on the ability of KG1-a cells to proliferate.

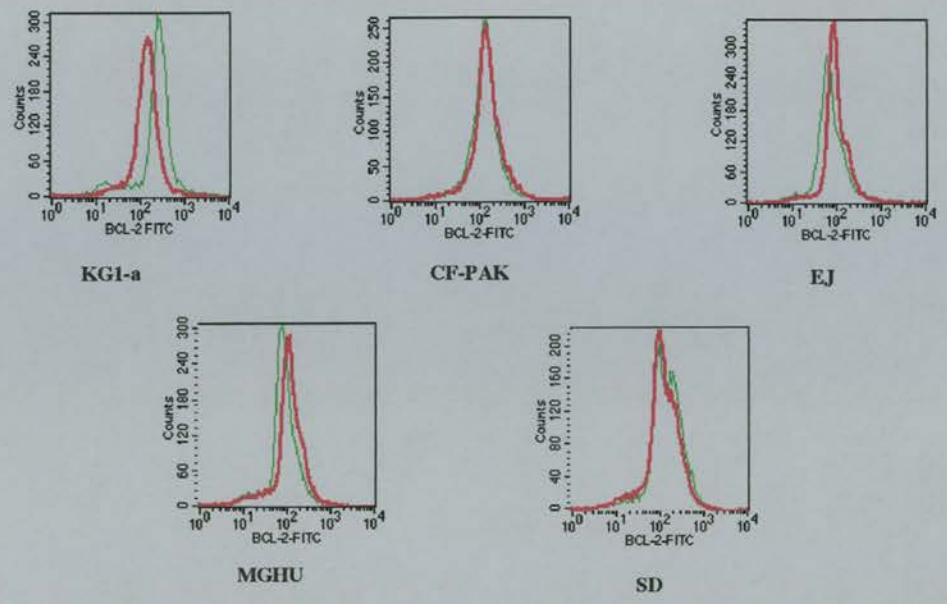
Effects on bcl-2 expression

The effects of antisense species on bcl-2 protein expression were quantified on the KG1-a (acute myeloid leukaemia) cell line using the quantitative flow cytometric analysis described before. Similarly carcinoma cell lines EJ, MGHU, SD, CF-PAK were measured for the effects of antisense on bcl-2 expression (**Figure 28**). Briefly cells (10^6/ml) were incubated in the presence of various antisense moieties for 1-7 days and the cells harvested, washed, permeabilised and stained for bcl-2 and DNA content. It was expected that there might not be gross induction of apoptosis but that there might be limited down-regulation of bcl-2 content wich in turn might lead to limited differentiation of these cells since more mature myeloid cells have been shown to be associated with a lower bcl-2 content (Porwit-McDonald et.al.1995). There was no obvious reduction in bcl-2 content except a small reduction in total bcl-2 content seen occasionally with KG1-a cells which did not reflect any development of apoptosis, similar to findings by other authors (Keith et.al.1995).

Antisense effects in the presence of cytotoxic agents

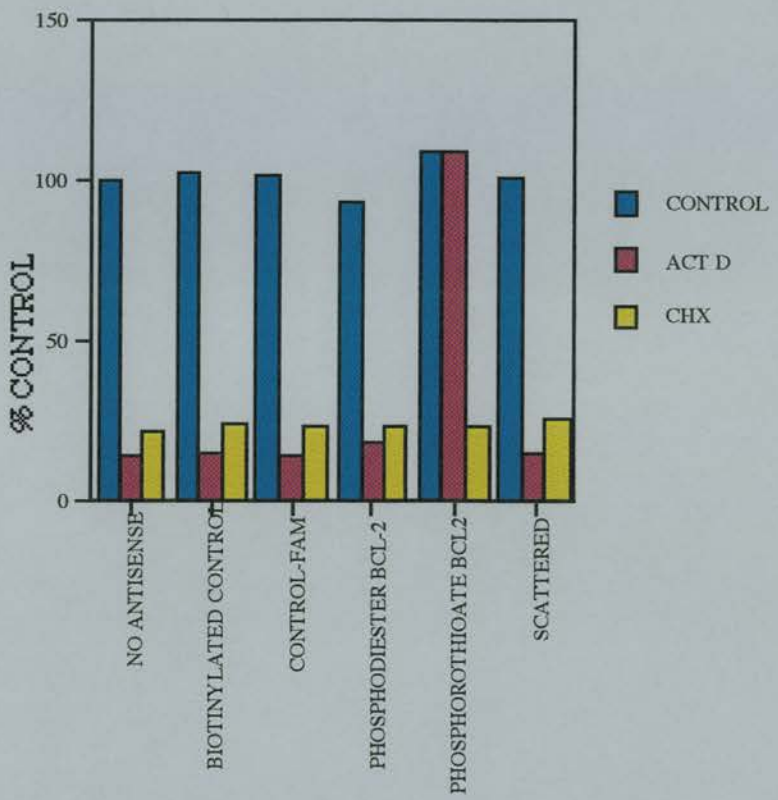
Given the poor effects of bcl-2 antisense on cellular viability on its own, the synergistic effects of antisense in the presence of known cytotoxic agents was studied. Cytosine arabinoside (Ara C) was chosen since it is of therapeutic clinical use in the treatment of leukaemias, particularly in relapsed and resistant forms. It uses the equilibrative nucleoside transporter (up to 24,000 receptors per AML cell) to enter cells before conversion to the active triphosphate Ara CTP and has its effect on rapidly proliferating tumours (Wiley, 1997). Actinomycin D (ActD) another potentially useful clinical agent (although now surpassed by more effective agents) is known to intercalate into DNA backbones and prevent *de novo* mRNA translation. Cycloheximide (CHX) is widely used as a research tool for its ability to prevent *de novo* protein synthesis. It was hoped that CHX might mimic or interfere with antisense-specific effects on mRNA inhibition because of its similarity of action. ActD and CHX are not thought to be cell-cycle specific drugs whereas AraC is S-phase specific. In some cell types apoptosis is not dependent on protein synthesis but in others it can be blocked by Cycloheximide or Actinomycin D (Arends et.al.1991). Others have found there was no correlation between the metabolic activity of AML blasts compared with their proliferative capacity (Elgie et.al.1996). The less active cells however appeared to be more sensitive to 6-thioguanine and more active cells to cytosine arabinoside. These agents however have all been described to induce apoptosis in many cell systems and are kown to induce apoptosis in KG1-a cells (AC unpublished data).

Antisense phosphorothioate Bcl-2 effects on tumour cell lines



(**Figure 28**). Flow cytometric histograms demonstrating the effects of bcl-2 antisense on bcl-2 protein expression in tumour cell lines. A small reduction in bcl-2 expression is seen with KG1-a but did not result in induction of apoptosis (green -control) (red antisense bcl-2).

POTENTIATING EFFECTS OF ANTISENSE ON CYTOTOXIC AGENTS



(**Figure 29a**). No potentiating effects of antisense were seen in the presence of Cycloheximide or Actinomycin D. However antisense bcl-2 phosphorothioate ameliorated the cytotoxic effects of Actinomycin D.

Following determination of an approximate LD_{50} for each of these drugs (ActD 4μM, CHX 50μM, AraC 1μM) using the MTT viability assay cells were incubated in the presence and absence of 5μM antisense bcl-2 (phosphorothioate and phosphodiester), control antisenses (biotinylated control and unlabelled nonsense (scattered) sequence) in the presence and absence of drug (**Figure 29**).

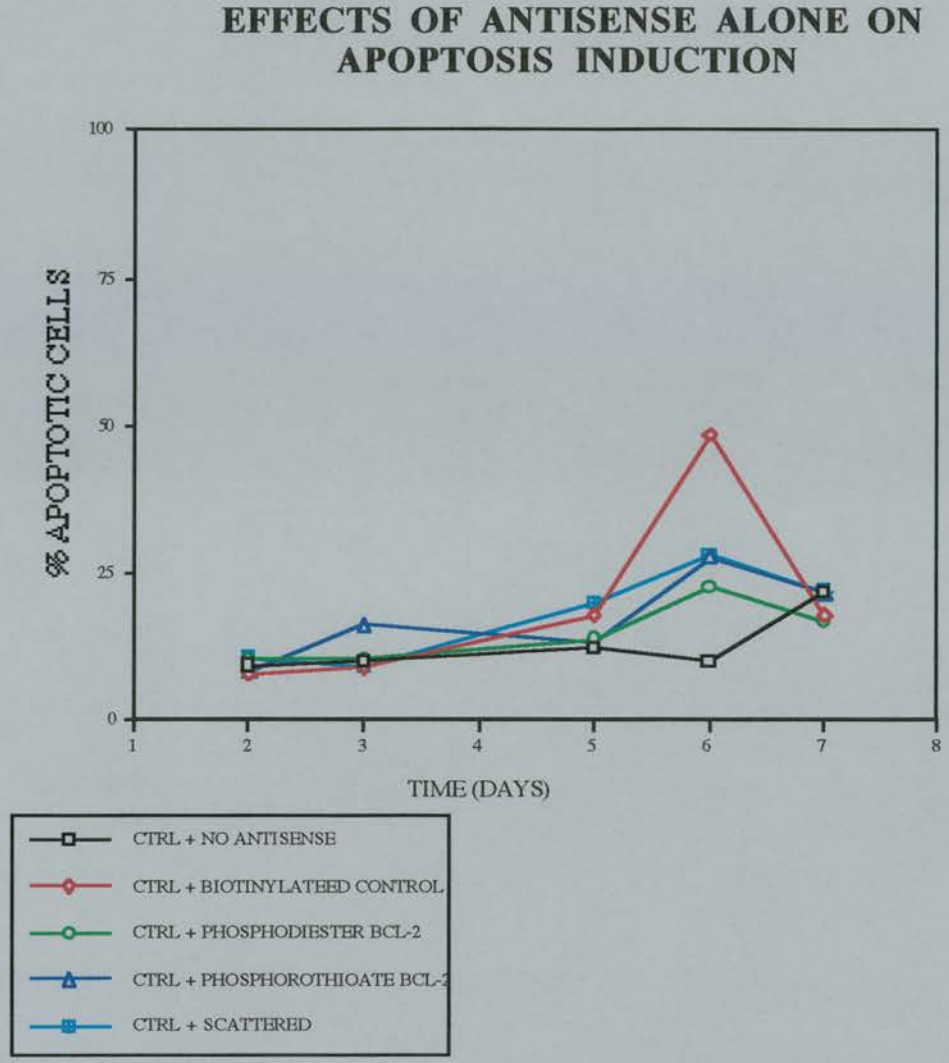
10^6 cells were incubated in the presence or absence of various antisense moieties for 1-7 days and the cells harvested, washed, permeabilised and stained for total bcl-2 and DNA content. A parallel sample was harvested and stained with Annexin V-FITC and propidium iodide to measure the functional effectiveness at inducing apoptosis. All conditions were measured by flow cytometry and evidence of apoptosis confirmed by microscopy.

An MTT cell proliferation assay of duplicate samples of the above conditions in the presence and absence of antisense revealed there to be no obvious synergy of effects on cell proliferation at 7 days post incubation (2 day antisense alone followed by 5 days antisense + drug) (Kitada et.al.1994). However there appeared to be an interesting anomaly in that antisense bcl-2 phosphorothioate appeared to abrogate the cytotoxic effects of Actinomycin D (**Figures 29a and d**). This effect was not seen with the same DNA sequence antisense bcl-2 phosphodiester.

The cells were therefore studied by flow cytometry in a time-dependent fashion to see if the pattern could be replicated. Inhibitors of cell proliferation (eg. dibutyryl cAMP, azide, amsacrine, mitomycin C, cycloheximide all of which reduce tritiated thymidine (^3H)-uptake have ben shown to to affect MTT and in some cases increase the signal. MTT has been shown to be more closely linked to glycolysis and drugs which affect glucose transport (Berridge et.al.1996). However it is clear that this is not the case in this study and all data was confirmed by flow cytometric detection of bcl-2 content and apoptosis induction by the Annexin assay.

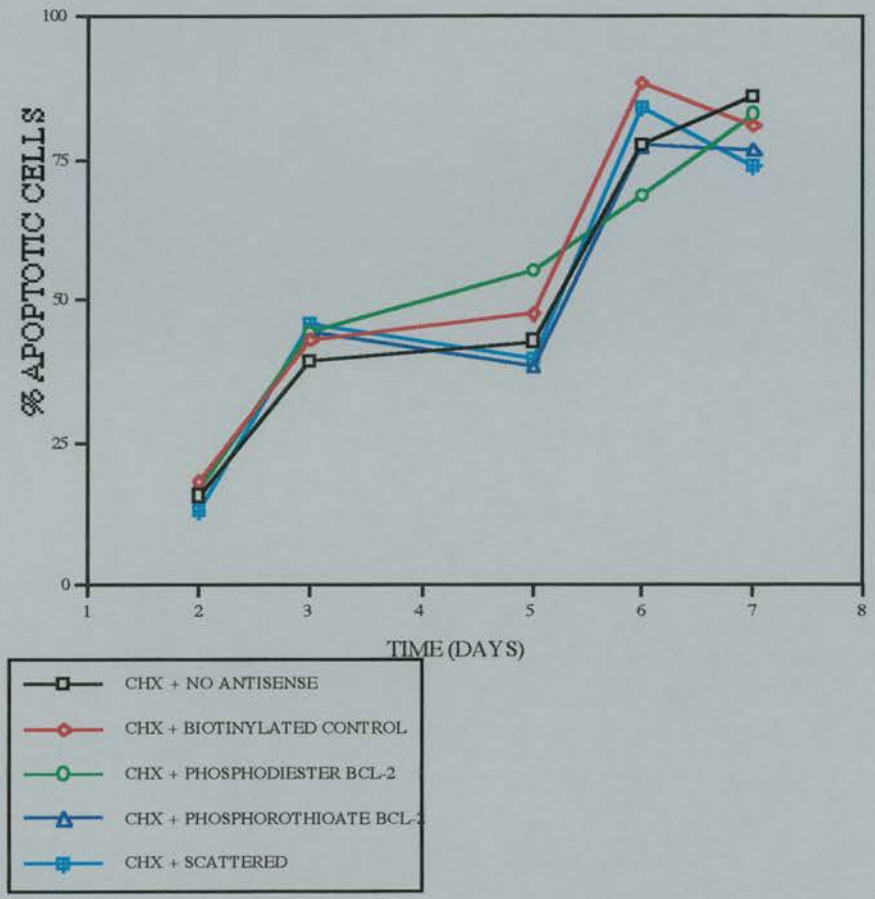
Flow cytometry revealed similar patterns of bcl-2 expression as expected from the MTT assay viability results. As previously described, apoptotic cells display a characteristic bi-phasic bcl-2 expression with the lower expression representing apoptotic cells and the higher peak representing viable cells (**Figure 30**). Therefore an increase in the percentage cells in the apoptotic lower bcl-2 content peak was used as a measure of the functional effectiveness of the antisense.

Cells treated in the absence of cytotoxic drugs there was a similar response to all antisense species. There was no obvious difference from untreated control cells except at six days when there seemed to be a marked elevation in bcl-2 levels with all antisense compared with the control (**Figure 29a and b**). The reason for this is unclear but may represent a non-specific toxicity because of nutrient loss and natural pH reduction or a reduced pH caused by the acid DNA rather than any true antisense-mediated effect.



(**Figure 29b**). KG1-a cells treated in the presence of antisense and absence of cytotoxic agents show no induction of apoptosis.

SYNERGISTIC EFFECTS OF ANTISENSE AND CYCLOHEXIMIDE ON APOPTOSIS INDUCTION

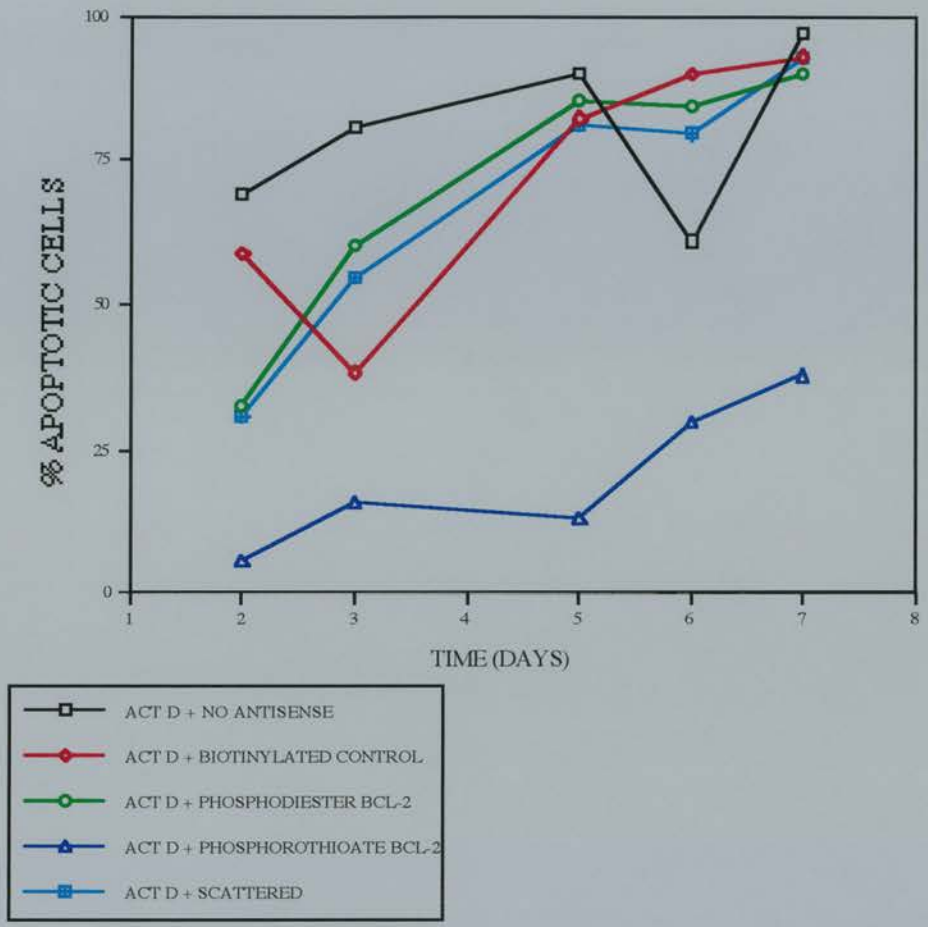


(**Figure 29c**). KG1-a cells treated in the presence of antisense and Cycloheximide show no potentiation in the induction of apoptosis.

In cells treated with Cycloheximide there did not appear to be any synergistic effect of antisense on apoptotic low bcl-2 cell populations (**Figure 29b**). It appeared therefore that apoptosis in these cells is not dependent on *de novo* protein synthesis. It is more likely that a protein phosphorylation-dephosphorylation signal forms the major apoptotic stimulus in these cells. Cycloheximide may also affect changes in the relative half-lives of essential proteins required for viability such as bcl-2. An increase in the relative concentration of a pro-apoptotic member of the bcl-2 family may therefore induce apoptosis.

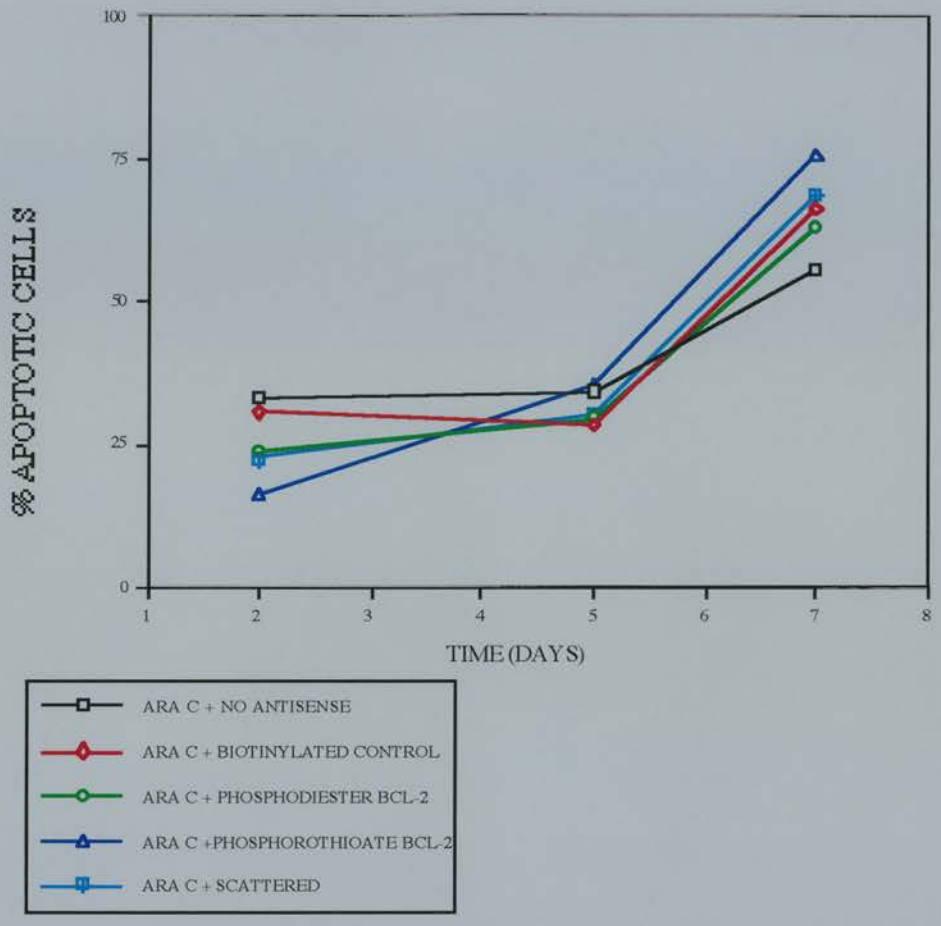
Cells treated with Actinomycin D showed a similarly high level of apoptosis in control cells. At early time points there appeared to be some protective effects of antisense on cellular viability with all antisense species though this effect was abrogated by day 5 post culture. It is unclear through what mechanism this may be acting, potentially the antisense might block entry of Actinomycin D to the cells at early time points an effect which is overcome as intracelluar levels of ActD rise over time (**Figure 29c**) and levels of phosphodiester antisense molecules decrease. However in cells treated with a combination of Actinomycin D and antisense bcl-2 phosphorothioate the cytoprotective effect is most apparent with only a fraction of cells undergoing apoptosis under these conditions. ActD is known to readily enter cells and to bind to double stranded DNA at 2 coincident G-C pairing regions. This might be a possible explanation for the cytoprotective effects of antisense bcl-2 phosphorothioate. To address this possibility samples of antisense were electrophoresed on an 18% polyacrylamide gel to look for differences in migration patterns (**Figure 32**). This revealed that the antisense bcl-2 phosphorothioate did in fact have a different migration pattern to that of the native bcl-2 phosphodiester moiety. This might be related to secondary structure formation such that several of the antisense molecules might homodimerise. In certain species this would give rise to double stranded DNA molecules containing the prerequisite Actinomycin D binding sites. Each antisense dimer would then have the capacity for binding two molecules of ActD (**Figure 31**) effectively abrogating its cytotoxic action. Polyacrylamide gel electrophoresis revealed a difference in migration pattern between the bcl-2 antisense species. The phosphodiester moiety migrated further than the phosphorothioate species indicating there were differences in structure between the two molecules (**Figure 32**). Whether this structural difference is sufficient to account for the differing actions in the presence of Actinomycin D is unclear.

SYNERGISTIC EFFECTS OF ANTISENSE AND ACTINOMYCIN D ON APOPTOSIS INDUCTION

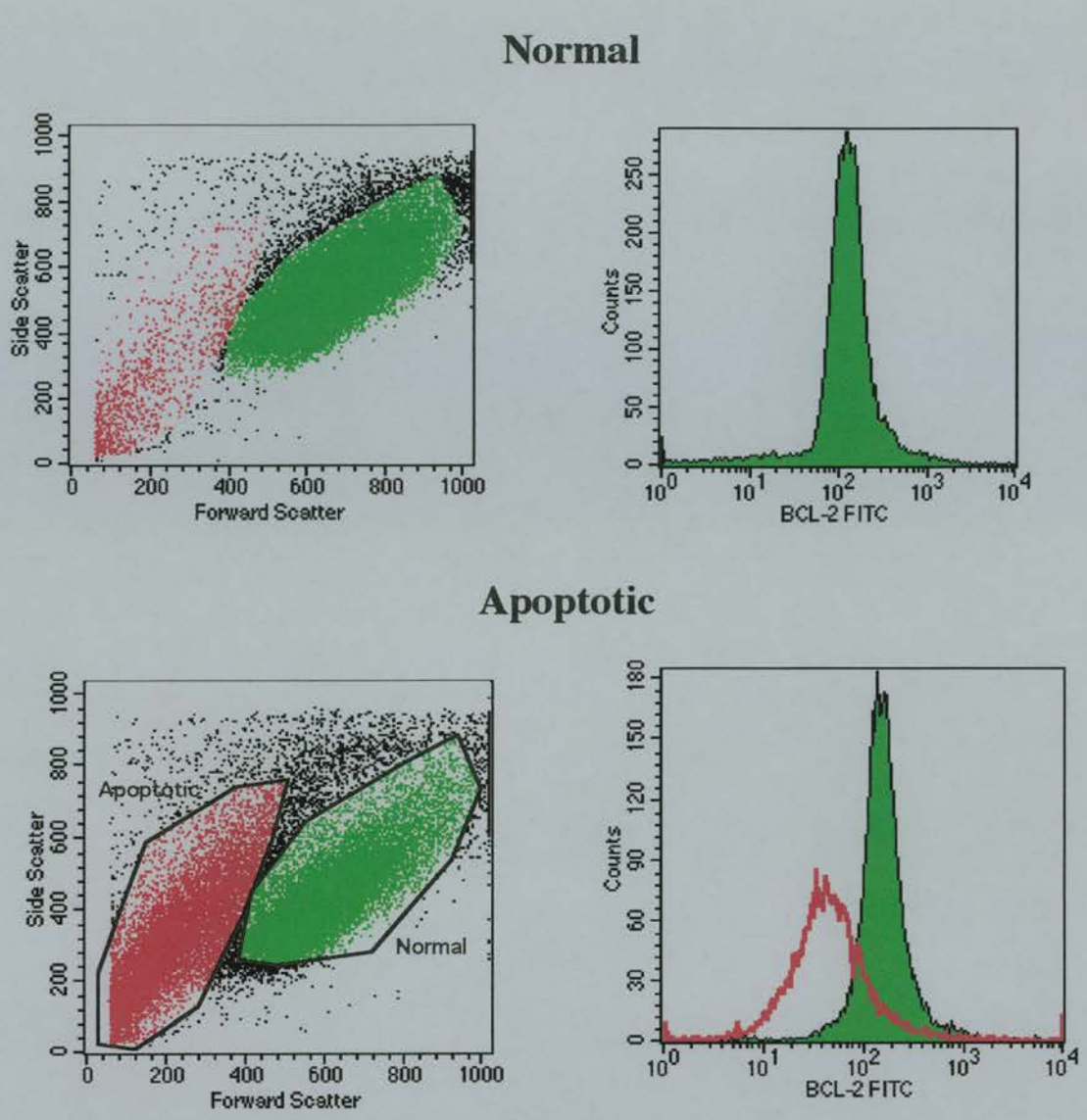


(**Figure 29d**). KG1-a cells treated in the presence of antisense and Actinomycin D show no potentiation in induction of apoptosis. However 5μM bcl-2 antisense phosphorothioate is able to suppress the induction of apoptosis in these cells.

SYNERGISTIC EFFECTS OF ANTISENSE AND CYTOSINE ARABINOSIDE ON APOPTOSIS INDUCTION



(**Figure 29e**). KG1-a cells treated in the presence of antisense and cytosine arabinoside show a small potentiation in induction of apoptosis with all antisense species. However 5μM bcl-2 antisense phosphorothioate is able to the increase the induction of apoptosis in these cells 50% more than cells with no antisense.



(**Figure 30**). These flow cytometric dot plots and histograms graphically demonstrate the reduced bcl-2 content of apoptotic cells. This represents a log reduction in bcl-2 content between viable and apoptotic cells. This feature was found in all cells studied though it is unclear whether the low bcl-2 content is an initiating event or a secondary event in apoptosis induction.

Potential antisense secondary structure formation

Bcl-2 antisense sequence: A) Partial Annealing

```
5' - TCTCCCAGCGTGCGCCAT - 3'
                  | | | |
          3' - TACCGCGTGCGACCCTCT - 5'
```

B) Hairpin Loop formation

```
      5' TCTCCCAGCGT
                 | | |
       3'      TACCGCG
```

Scattered (nonsense) sequence

A) Partial Annealing

```
5' - TGCACTCACGCTCGGCCT - 3'
                  | | | |
          3' - TCCGGCTCGCACTCACGT - 5'
```

OR

```
                  5' - TGCACTCACGCTCGGCCT - 3'
                       | | | |
3' - TCCGGCTCGCACTCACGT - 5'
```

Control antisense sequence has no obvious secondary structure formation.

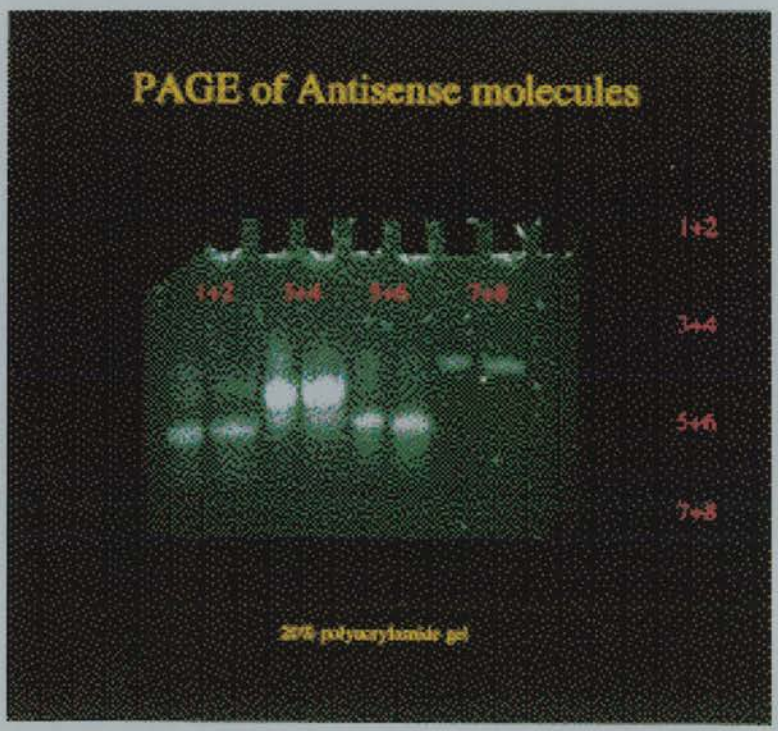
5' - CTCTCGCACCCATCTCTCTCCTTCT - 3'

(Figure 31)

This antisense-Actinomycin complex may be sequestered into cytoplasmic vesicles and reduce the bioavailbility of Actinomycin D. E.coli single-stranded DNA binding protein was used at a molar ratio of 0.7 ssDNA to SSB (Bleiberg et.al.1981) in an attempt to reduce this effect but was not found to be effective at increasing antisense uptake in KG1-a cells (**Figure 25**).

Cells treated with cytosine arabinoside showed a small but discernible synergistic effect with all antisense molecules studied with the greatest effect seen with antisense bcl-2 (**Figure 29d**). It is known that cytosine arabinoside can cause cell membrane fragility (White et.al.1987; Rathmell et.al.1986). AraC may therefore disrupt the membrane barrier and allow antisense access to the cytoplasmic mRNA target whereas normally it is sequestered in vesicles. This might allow for specific hybridisation to target sequences and thus exert a true antisense-mediated effect.

There may also be possible non-specific antisense-mediated effects such as non-specific protein binding and antisense sequestration to the nucleus (Leonetti et.al.1991) where again effects on histones, transcription factors may all exert non-specific anti-proliferative effects (Stein, 1995).



(**Figure 32**). This polyacrylamide gel (20%) demonstrates the different migration patterns between bcl-2 phosphodiester (lanes 1+2) and bcl-2 phosphorothioate (lanes 3+4) antisenses. Nonsense (scattered) antisense (lanes 5+6) and biotinylated control antisense are also shown.

Mac-1 - an Antisense Receptor ?

A previous study by (Neckers, 1993) have described a putative antisense receptor to be an 80kDa cell membrane protein, recent publications however have suggested that antisense uptake may be associated with the CD11b-CD18 (Mac-1) integrin. This molecule is a receptor for many ligands including complement C3, ICAM-1, and fibrinogen (Diamond et.al.1995). Mac-1 contains a number of binding sites including a divalent cation-dependent-adhesion-site and heparin-binding sites. CD11b has relatively limited lineage-restricted expression and is normaly found on neutrophils, monocytes and some NK cells. In their resting states there are differences in the levels of expression with monocytes having greater levels than neutrophils which in turn have higher levels than NK cells. Basal levels may be up or down-regulated by various cytokines or pharmacologic agents.

Peripheral blood

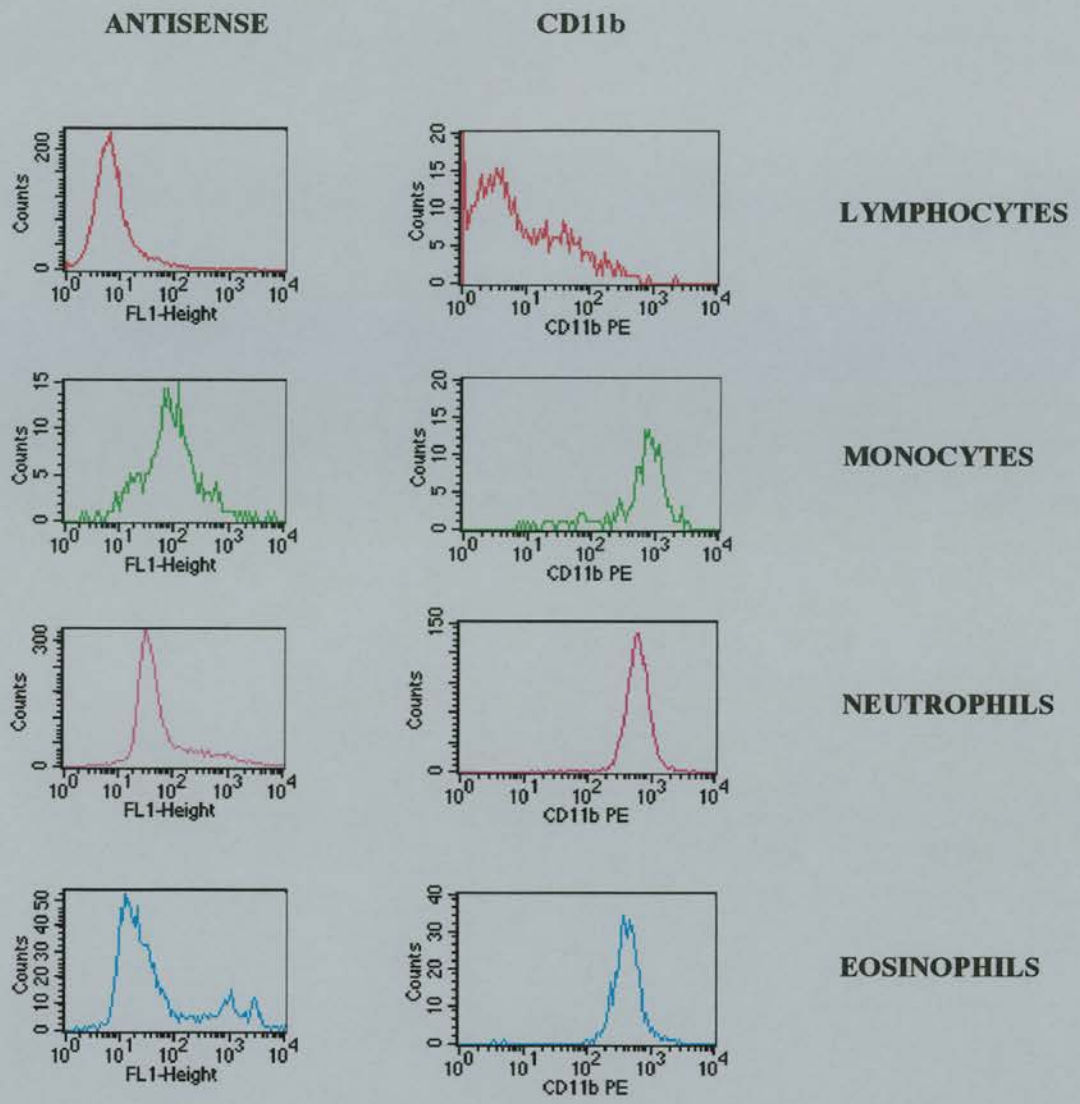
Therefore normal peripheral blood CD11b levels were characterised in samples analysed by flow cytometry using a PE labelled CD11b monoclonal antibody (Leu-15a Becton Dickinson, Oxford UK). CD11b levels in normal peripheral blood were determined and were found to be similar (in levels of expression) to the patterns of antisense uptake found in these cells. (**Figure 33a and b**). It therefore appeared as though antisense uptake might be dependent on some characteristc of the Mac-1 molecule. The most likely epitope was likely to be a heparin-binding site since antisense molecules are highly charged acidic molecules (Bleiberg et.al.1981). Antisense DNA backbones are heavily negatively charged because of the acid phosphate groups and are therefore attracted to non-specific positively charged sites. Antisense phosphorothioate molecules also structurally resemble natural heparins in their degree of linearity and sulphation.

KG1-a cells

Similarly KG1-a cells were stained with anti-CD11b antibody and approximately 10% of the cells were found to express this marker but were otherwise phenotypically normal. KG1-a cells had a typical immunophenotype of CD34, 7, 71, 45, 95 positive, CD.19, 41a negative, with varable amounts of CD33 and 38. They were also known to express the following adhesion moecules CD 54, 31, 58, 49d, 49c 11a, 44, 36.(Dr M. Turner personal communication). The levels of uptake of 5μM antisense-FAM were studied at 5 and 10 hours in the CD11b positive and negative fractions of KG1-a cells simultaneously (**Figure 34**). It was found that higher levels of antisense were found in CD11b positive cells at 5 and 10 hours and somewhat lower levels found in CD11b negative KG1-a cells. By 10 hours the extracellular levels in CD11b positive and

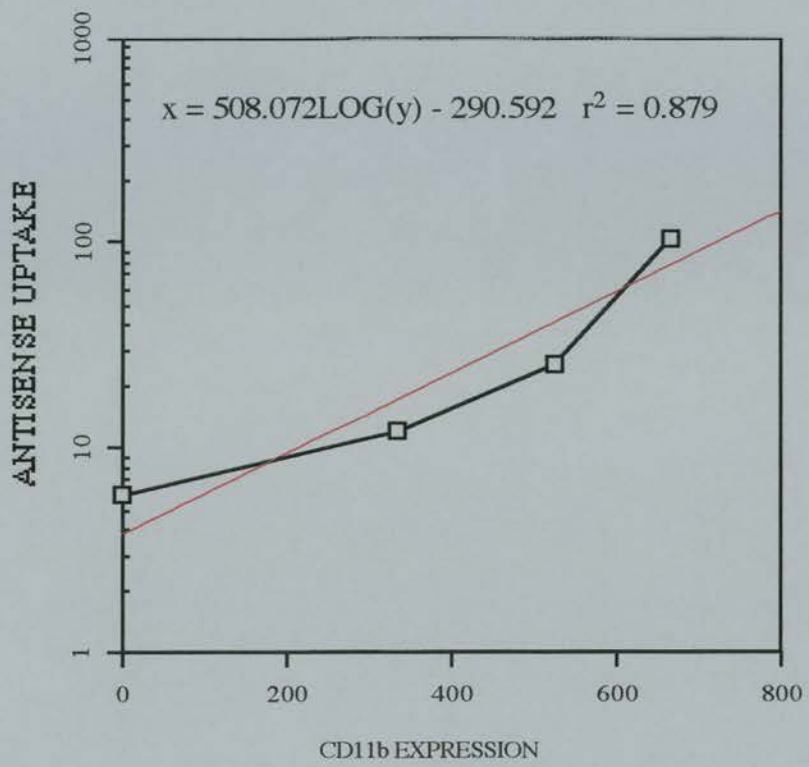
negative cells had risen to levels found inside the cell indicating equilibrium had been reached in both cases although at differing intensities. This may be due to differing levels of similar equilibrative transport systems.

The expression of CD11b was determined in conjunction with levels of 5μM antisense uptake in CD11b positive KG1-a cells and it was found that there was an inverse relationship between the two. As antisense levels increased the cellular CD11b content decreased at a similar rate indicating that the antisense was triggering a down-regulation of CD11b expression in these cells (**Figure 35**). This can be most readily explained by antisense binding to CD11b and being internalised prior to antisense compartmentalisation into cytoplasmic vesicles. These vesicles are a feature of many receptor-mediated ligand internalisation processes such as that described for transferrin (Cotten et.al.1990; Zenke et.al.1990; Wagner et.al.1990) and are readily apparent under UV microscopy and have been described by other authors (**Figure 24**).



(**Figure 33a**). This figure shows the similarity between patterns of cellular antisense association and the CD11b expression on cell types of normal peripheral blood.

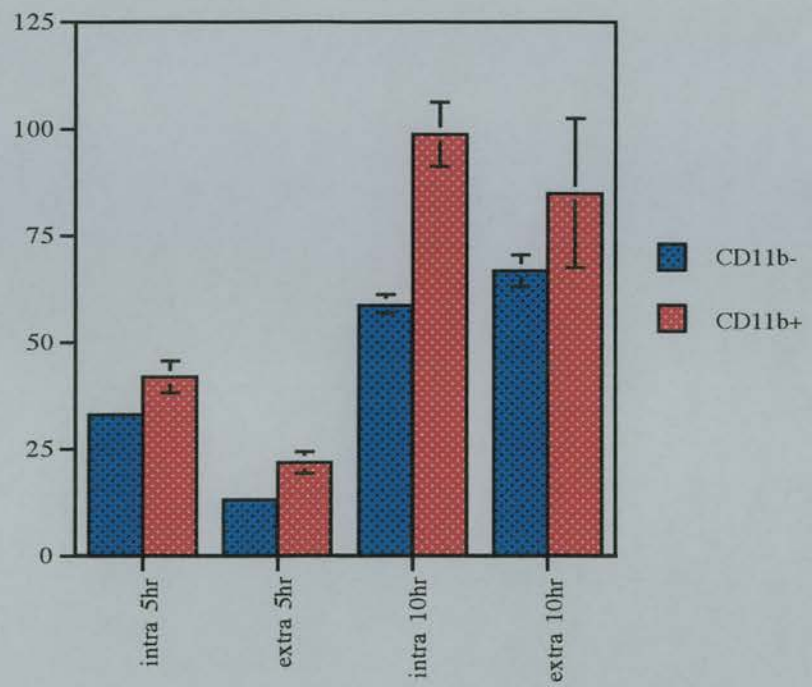
RELATIONSHIP BETWEEN CD11b EXPRESSION AND ANTISENSE UPTAKE



(Figure 33b)

(**Figure 33a and b**). These figures demonstrate the similarity between patterns of cellular antisense association and the CD11b expression on cell types of normal peripheral blood.

ANTISENSE LEVELS IN CD11b POSITIVE AND NEGATIVE KG1-a CELLS



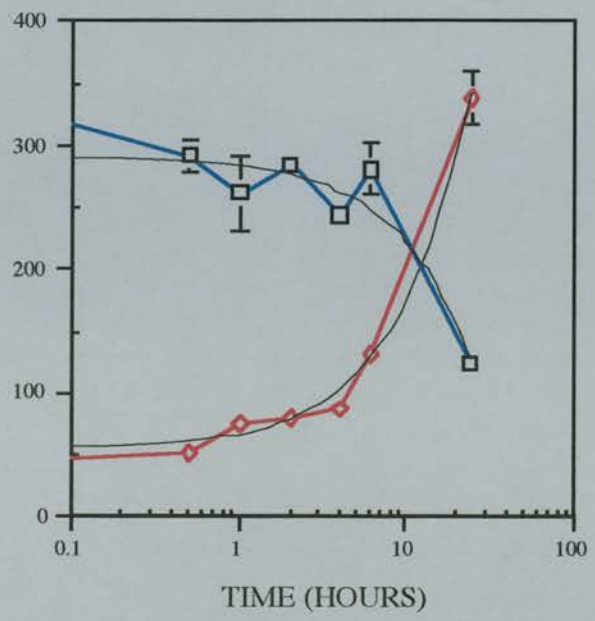
(**Figure 34**). This figure demonstrates that within a single cell line (KG1-a) those cells which express the adhesion molecule CD11b have a greater antisense association than those which do not, with almost a doubling of the concentration inside the cell at 10 hours.

Inhibition of antisense uptake

The evidence that CD11b was a possible antisense receptor suggested that a heparin-binding site was the most likely epitope. To address this possibility 5μM antisense was co-incubated with increasing doses (0-100 IU/ml) of low-molecular weight heparin (Pump-Hep, Leo Laboratories, UK). The fluorescent antisense signal was measured in CD11b positive and negative KG1-a cells. There was an obvious inhibitory effect of heparin on antisense uptake in both cells (**Figure 36**). However the rate of inhibition appeared greater in CD11b positive (slope -32) than CD11b negative (slope -12) cells.

The effects of polyanions were studied in KG1-a cells (**Figure 37a**) and compared to agents which are known to affect CD11b and or bcl-2 expression in HL-60 promyelocytic leukaemia cells were studied (**Figures 37b,c**). DMSO and retinoic acid are known to drive HL-60 towards neutrophilic cells with a concomitant reduction in bcl-2 content and up-regulation of CD11b. PMA, a phorbol ester, is known to drive these cells towards monocyte-like cells but with lesser effects on CD11b expression. The polyanionic effects of heparin and antisense were thus compared for effects on CD11b and bcl-2 content. Heparin and control antisense showed similar effects in down regulating CD11b expression in KG1-a cells. Levels were reduced to approximately 60% of control cells in the presence of 100 IU/ml heparin or 5μM control antisense indicating a likely polyanionic effect. In HL-60 cells DMSO heparin, and bcl-2 antisense increased the percentage of CD11 positive cells whereas PMA showed a slight reduction. The intensity of CD11b expression was down-regulated by PMA, DMSO and retinoic acid and to a lesser extent with heparin and bcl-2 antisense. However the control antisense had a marked elevation of CD11b intensity indicating a possible triggering of CD11b signalling mechanisms which up-regulate levels of this integrin.

RELATIONSHIP BETWEEN ANTISENSE UPTAKE AND CD11b EXPRESSION IN KG1-a CELLS

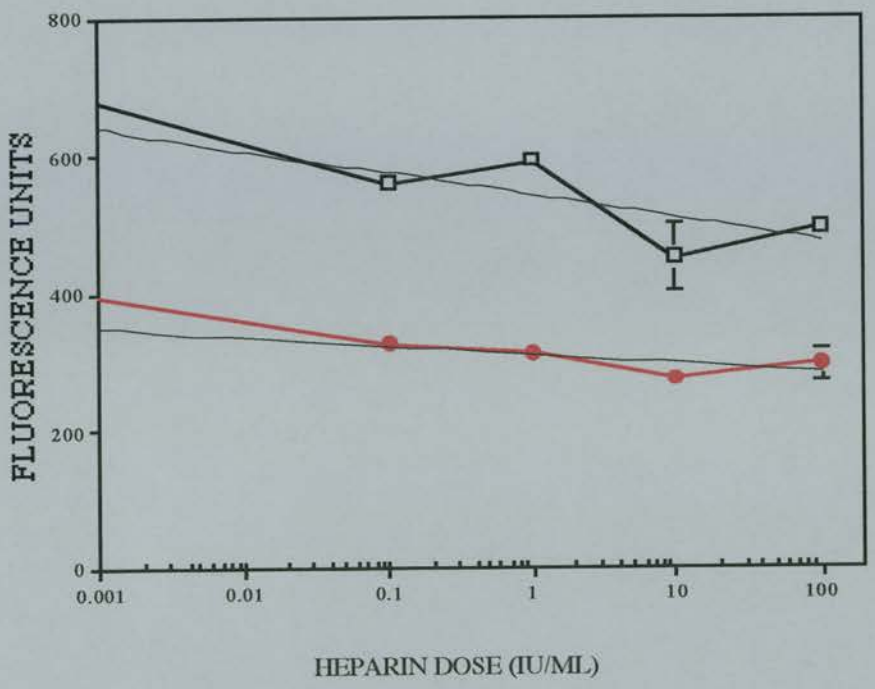


CD11b expression $y = -6.696x + 288.561$ $r^2 = 0.903$

Antisense-FAM uptake $y = 11.882x + 52.960$ $r^2 = 0.993$

(**Figure 35**). Showing the inverse relationship between antisense uptake and CD11b expression. As antisense is taken up into cells the CD11b expression drops, suggesting a receptor-mediated internalisation or competition for epitopes.

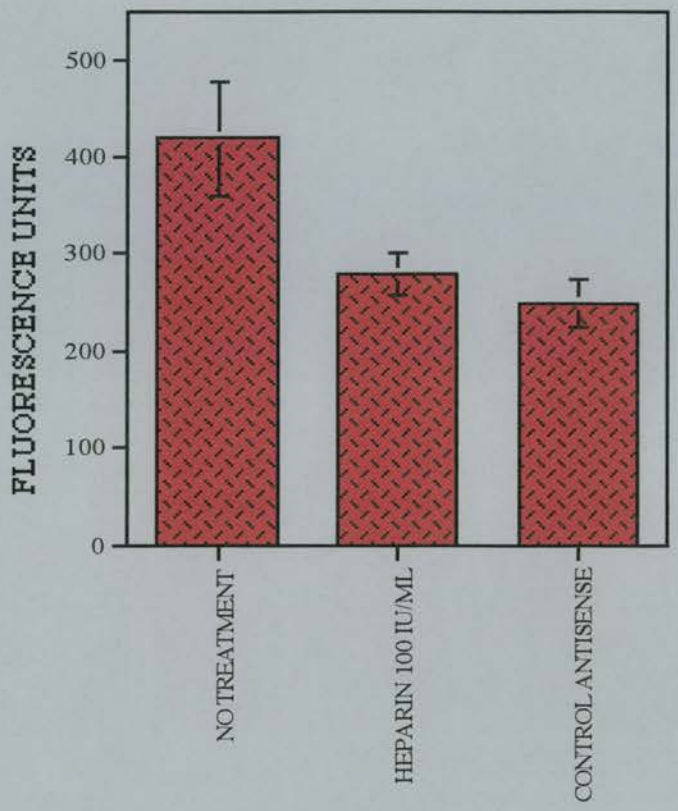
INHIBITORY EFFECTS OF HEPARIN ON ANTISENSE UPTAKE OI
CD11b POSITIVE AND CD11b NEGATIVE KG1-A CELLS



(**Figure 36**). This graph shows the differential inhibition of heparin on antisense uptake into CD11b positive and negative KG1-a cells.

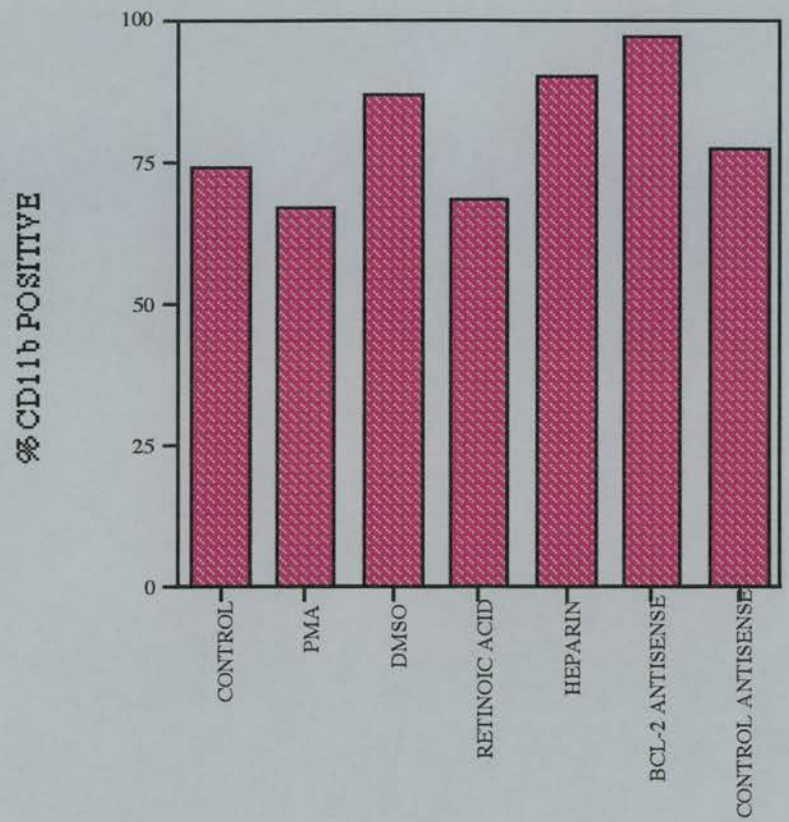
Bcl-2 expression appeared to be less affected by any of the treatments with only a slight reduction in intensity seen with any of the treatments.
Similarly because of the many ligands for Mac-1, including fibrinogen, the uptake of antisense in the presence and absence of serum was monitored. KG1-a cells were incubated with 5μM antisense in QBSF-51 serum-free medium for 5 hours in the presence and absence of 10% foetal calf serum (**Figure 38**). Levels of antisense were greater in serum-free medium indicating the presence of an inhibitory component of serum. This is unlikely to be caused by fibrinogen because it is removed during preparation of the serum, it may however be due to complement components (known to bind Mac-1) or non-specific binding components of serum such as albumin or alpha 2-macroglobulin which are known to bind small molecules.

CD11b EXPRESSION IN KG1-a CELLS



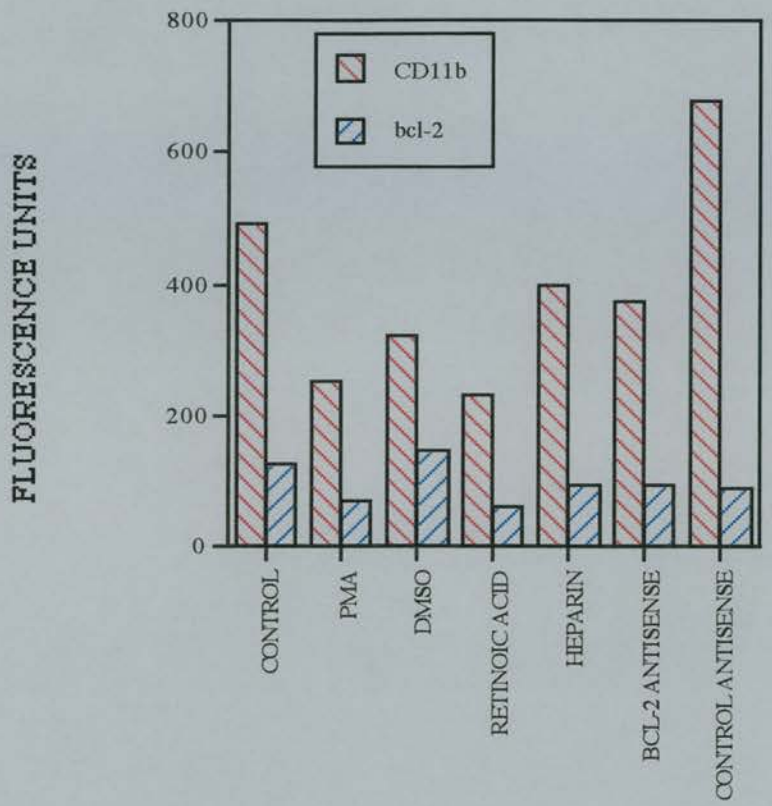
(**Figure 37a**). This graph shows the similarity of effects of the polyanionic molecules heparin (100 IU/ml) and control antisense (5μM) on the reduction of CD11b expression in KG1-a cells.

EFFECTS OF AGENTS ON CD11b EXPRESSION IN HL-60 CELLS

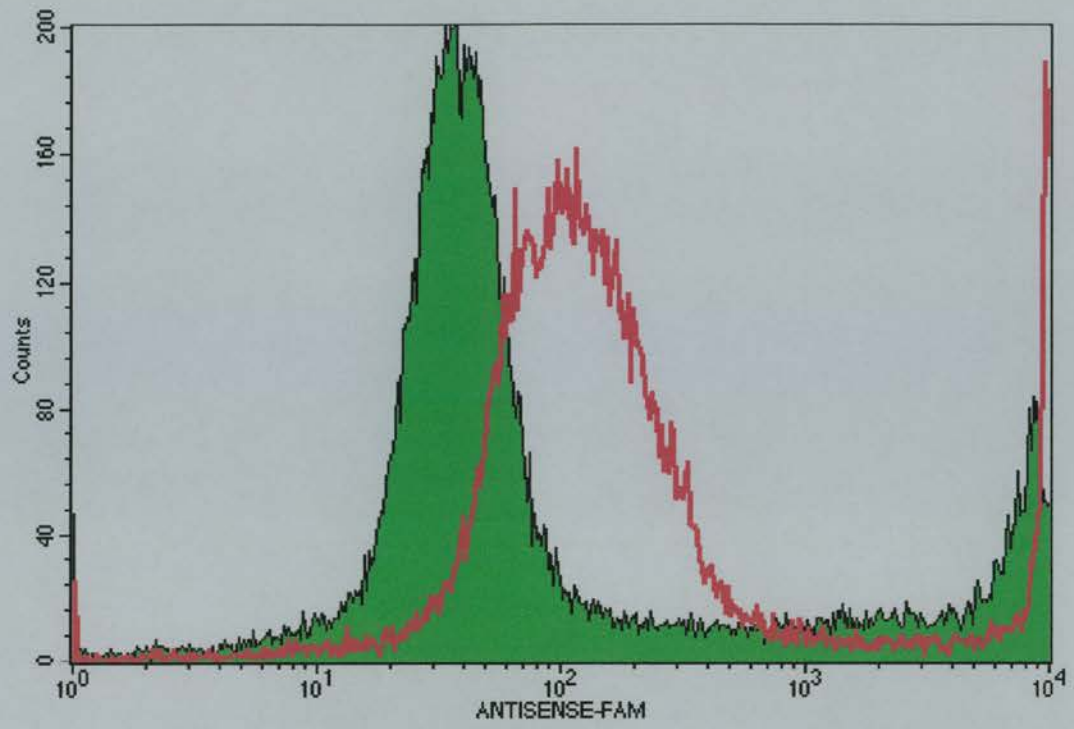


(**Figure 37b**). The effects of PMA, DMSO, Retinoic acid, heparin and antisense were studied for their ability to induce changes in the patterns of CD11b expression in the HL-60 promyelocytic leukaemia cell line. DMSO, heparin and antisense bcl-2 increase the number of CD11b positive cells, which is likely to represent a differentiation towards a neutrophilic lineage.

EFFECTS OF AGENTS ON CD11b AND Bcl-2 EXPRESSION IN HL-60 CELLS



(**Figure 37c**). The effects of a number of agents were studied for their ability to induce changes in CD11b and bcl-2 expression in HL-60 cells. PMA, DMSO, Retinoic acid are known to be capable of inducing differentiation in these cells and the effects polyanions (heparin & antisense) compared for their ability to regulate CD11b and bcl-2 expression.



(**Figure 38**). This flow cytometric histogram graphically demonstrates the inhibitory effects of serum on the uptake of 5μM antisense into KG1-a cells. Cells were incubated in the presence (green peak 43) or absence (red peak 76) of serum in QBSF-51 serum-free medium.

Summary

In summary this thesis set out to look at bcl-2 protein levels in normal and AML cells and relate these levels to colony growth in AML blast cells. Antisense was used in an attempt to reduce bcl-2 protein levels and increase the sensitivity of AML cells to chemotherapy to improve the prognosis for these patients.

This study found there was no overall elevation of bcl-2 level above normal cell types though individual patients had abnormally high levels. This was borne out by the poor ability of blast cells from these AML patients to form colonies *in vitro*. It was not possible however to monitor these patients' subsequent clinical progress.

Studies on the uptake of fluorescent antisense molecules described a discrete cellular partitioning with a high level of extracellular association and intracellular vacuolar location. This cellular association appeared to be linked with the β2 integrin Mac-1 (CD11b-CD18) expression on cell membranes. Higher levels of antisense uptake were found in cells expressing CD11b and could be inhibited with polyanionic molecules such as heparin.

This offers a potential *in vivo* therapeutic targetting mechanism of antisense molecules to cells which express this integrin (neutrophils, monocytes, NK cell and some AML blast cells). CD11b-expressing AML blast cells have similarly been associated with a poorer disease prognosis and may benefit most from this type of antisense approach.

Targetting of antisense molecules to peripheral blood cells may therefore also present a viable therapeutic option. Neutrophils are easily targetted with a short bolus of high concentration drug. Because of elevated levels of CD11b on neutrophils and their short life-span non-specific effects on other cell types could be minimsed. In the lung excess eosinophils are thought to be involved in the pathology of inflammatory lung disease. Antisense to pro-inflammatory mediators such as IL-5 could be administered as an aerosol and eosinophils targetted through CD11b expression.

Similarly with monocytes levels of CD11b could be up-regulated by systemic administration of TNF-α (unlikely because of toxic systemic effects) or GM-CSF thus increasing the therapeutic index of the drug (Ohsaka et.al.1997). This last option is of considerable interest in AML since those leukaemias which express CD11b are associated with a poor complete remission response and these patients are also associated with elevated levels of bcl-2.

One could therefore envisage therapeutic administration of growth factors (namely GM-CSF) to a) alleviate the neutropenia associated with chemotherapy, b) mobilise blast cells into cycle and increase their sensitivity to S-phase specific chemotherapy and c) upregulate levels of CD11b to allow more effective targetting of antisense bcl-2 to resistant tumour cells.

Further studies should confirm that antisense can exit from these vacuoles using lysosmotropic agents and is similarly effective at reducing the capacity of AML blast cells at surviving toxic insults. Furthermore alternative targets other than bcl-2 may be more efficacious at killing tumour cells.

Discussion

Bcl-2

Bcl-2 protein has been definitively located by electron microscopy to the outer mitochondrial, and the cytoplasmic side of nuclear and endoplasmic reticulum membranes and is intimately involved in the regulation of permeability pore transition and is found in many cell types (Yang et.al.1996). The t(14;18) chromosomal translocations which gives rise to elevated bcl-2 levels in lymphoma are often also found in normal healthy individuals' tonsils, or in peripheral blood. Also lymphomas without the chromosomal translocation similarly have elevated bcl-2 levels. Thus bcl-2 elevation through translocation is not sufficient for tumourigenesis. Elevated long-term expression of bcl-2 and suppression of apoptosis may allow accumulation of further DNA damage, perhaps as a result of the chemotherapy used to treat the original disease. Similarly loss of bcl-2 protein in breast cancer is correlated with a poorer prognosis (Yang et.al.1996). Bcl-2 expressing cells enter into G0 upon growth factor removal. A rise in p53 (due to DNA damage) causes a transient arrest in G1 (to repair DNA). This is facilitated by p53-induced p21 which in excess inhibits cyclin-cdk complexes which normally drive cellular progression through G1 and S phases (Cory, 1995). Bcl-2 is able to inhibit this p53-mediated apoptosis in some cell lines (Nunez et.al.1994), thus bcl-2 is intimately linked to cellular homeostasis as described above.

Antisense bcl-2

Bcl-2 is expressed in normal haematopoietic cells including bone-marrow blasts promyelocytes and also malignant AML and CML blasts. Up to 70% of AML blasts have been shown to have autonomous growth and express increased bcl-2 which is linked to a poorer response to chemotherapy (Campos et.al.1993; Lowenberg et.al.1993). Therefore bcl-2 would appear to be an important therapeutic target especially in the more aggressive forms of AML. Downregulation of bcl-2 by pharmacologic methods should provide a single, reliable method applicable to most or all forms of refractory or resistant AML.

The exquisite theoretical specificity of antisense DNA should allow precise inhibition of single tumour-specific proteins. This type of technology has been shown in many cell free and *in vitro* systems to provide the specificity required but is limited at present by kack of an efficient specific *in vivo* delivery system (Zamecknik et.al.1986; Spiller et.al.1995; Leonetti et.al.1991; Fisher et.al.1993b). However many studies on primary clinical material have provided a more optimistic therapeutic potential. The study monitoring group 3 and 4 patients (those with partial or complete autonomous growth)

found that group 3 had a better (50%) response rate to antisense whereas group 4 patients had only 14% response. Patient samples and HL-60 cells required a dose of 5μM antisense for 3 days before any effects on bcl-2 were observed, presumably because of vacuolar partitioning (Keith et.al.1995). Antisense treatment on its own failed to indude apoptosis but increased the sensitivity to cytosine arabinoside, similarly in a previous study this group were able to reduce GM-CSF production with antibody treatment but this did not induce apoptosis either.

Bcl-2 is found to be expressed in normal myeloid and cell lines (such as KG1-a, K562, HL-60) and in the mantle zone of lymph nodes and in peripheral blood T and B cells (Campos et.al.1994; Porwit-McDonald et.al.1995). The highest levels were found in growth-factor independent cell lines. This is similar to the data found in this thesis with highest levels found in KG1-a cells. No effect of antisense was seen until day 11 using 25-50μM antisense, with only a 30% cell survival using 50μM drug. Again two peaks of bcl-2 expression were seen on flow cytometry indicating the lower bcl-2 content of the apoptotic cell population (**Figure 30**). Whether the reduction in bcl-2 is secondary to the initiating apoptotic signal, or whether reduced bcl-2 is part of the apoptotic signalling process is however unclear. The effectiveness of a 4μg/ml dose of cytosine arabinoside was shown to be more effective in the presence of antisense than sense or control molecules. According to this paper the highest intracellular antisense phosphorothioate content is achieved after 4-5 days which accounts for the delay in its functional effectiveness(Campos et.al.1994).

Chemotherapeutic agents induce apoptosis in highly-replicative target cells. Apoptosis is not cell cycle specific but the action of the drugs is. As well as increasing the sensitivity to these drugs antisense bcl-2 has been shown to accelerate death in pre-B cell leukaemias, T-ALL, NHL and AML. The normal half-life of that of bcl-2 mRNA approximately 3 hours whereas bcl-2 protein is more than 10 hours, but this may be altered by molecules such as bag-1, bax, bcl-x, mcl-1 (Reed, 1996).

Liposomal transfection of 75-300nm antisense or an inducible plasmid containing bcl-2 antisense, gave a reduction of bcl-2 mRNA within one day in SUDHL-4 t(14;18) lymphoma cells, protein levels however took about 3 days to show an effect (Kitada et.al.1994). Antisense had little or no effect on growth in the first three days and antisense-mediated reductions in bcl-2 protein levels (as in other cells) does not accelerate cell death unless the cells are deprived of growth factors or there is another pro-apoptotic stimulus. Cells treated for two days with antisense then exposed to cytotoxic drugs showed approximately an 80% increase in cytotoxicity over that seen with antisense treated cells alone or than control oligos plus drug. Antisense bcl-2 increased sensitivity of cells to cytosine arabinoside and methotrexate but effects on

bcl-2 protein reduction in the absence of cytotoxic drug had no effects on proliferation or survival. However the addition of 10^{-7}M cytosine arabinoside to cells transfected with a metallothionein-driven bcl-2 antisense-containing plasmid inhibited growth. In those containing the plasmid, but not induced by heavy metals no decrease in cellular survival were seen (Kitada et.al.1994).

Other targets for antisense in AML

Other potential targets for antisense therapy in AML include antisense to c-myb (a transcription factor important in haematopoietic development) which has been shown to inhibit AML cell growth in K562 erythroleukaemia (M6) cells (Gewirtz, 1992), KG1a (M0-M1) cells but especially HL-60 (M3) cells. Antisense-treated HL-60 cells appeared to be arrested in G1 or at the G1-S boundary. Inhibitory effects of c-myb antisense were found to be greater in leukaemic than haematopoietic progenitors and CFU-L could be reduced by 75-80% (Gewirtz, 1992). Antisense to c-myc in HL-60 cells causes cells to undergo terminal differentiation though some continue to proliferate (Kirkland, 1994). HL-60 APML cells contains high levels of bcl-2 which decreases dramatically during differentiation induced by PMA and other agents. PMA causes HL-60 to stop proliferating and differentiate into macrophage-like cells then die. PMA (10ng/ml) induces differentiation into monocytic cells and up-regulates CD11b and down-regulates bcl-2 (Blagosklonny et.al.1996) though the bcl-2 protein down-regulation is not required for differentiation. The half-life of bcl-2 protein in HL-60 cells is approximately 20 hours but can be as much as 2 times this in drug-resistant forms (Blagosklonny et.al.1996). Normal granulocytes were found to be bcl-xl negative, while monocytes and macrophages were found to be positive. Treating HL-60 cells with 1μM retinoic acid differentiates towards granulocytes (bcl-xl negative) whilst treating with PMA differentiates cells towards the macrophage and monocyte lineage (bcl-xl positive). In both treatment regimens bcl-2 levels drop (Sanz et.al.1997) as the cells differentiate.

An antisense phosphorothioate to IL-1β converting enzyme (ICE) at 10-75μM was found to inhibit proliferation of peripheral blood and bone marrow leukaemic cells and inhibited leukaemic colony (CFU-L) formation. IL-1 has been shown to be important in initiating paracrine and autocrine colony growth (Stosic-Grujicic et.al.1995) which is also associated with high GM-CSF secretion and high bcl-2 levels. The Wilms Tumour (WT) gene product is found to be expressed in all of 96 patients with AML or CML and is another potential targe for antisense therapy in AML. There were no effects of a WT antisense on normal CFU-GM but leukaemic colonies were inhibited by antisense. A WT cDNA to the antisense sequence (driven by a CMV promoter) abrogated antisense efects as determined by flow cytometry (Yamagami et.al.1996)

thus definitively demonstrating an antisense-specific inhibition.

Antisense Uptake

The objective of this thesis was therefore to determine the characteristics of uptake of antisense molecules into AML and normal blood cells *in vitro* with a view to developing an efficient.purging or systemic therapy to destroy leukaemic AML cells based on antisense to bcl-2 protein. This included determination of cellular localisation, dose-concentration relationships and delivery dynamics of unmodified antisense phosphorothioate molecules. The data described a consistent dose and time-dependent uptake of antisense into peripheral blood leucocytes, and that this uptake is made up of an extracellular and intracellular compartment, which are both dose-dependent. There appears to be an association of antisense to cell-surface heparin-binding sites including CD11b which is in agreement with other authors findings (Bleiberg et.al.1981; Harenberg et.al.1995; Benimetskaya et.al.1997).

Phosphorothioate antisense oligos have been shown to localise in the acidic intracellular compartment in HL-60 cells. 80% of internalised phosphorothioate resided in a cellular compartment with a turnover time of 2-5 hours. Uptake of oligos plateau at 3-6 hours (Tonkinson et.al.1994) and at 4°C 90% of the oligo associated with cells is localised on the membrane. However at 37°C the intracellular concentration rose and plateaued at 1-2 hours in serum-free medium. This uptake of antisense oligos was found to be taken up in a sequence independent manner (Neckers, 1993).

U937 cells (a diffuse histiocytic lymphoma cell line with monocyte-macrophage characterisics) were treated with c-myc antisense by electroporation which appeared to cause rapid cell death (presumably through an antisense effect). This treatment appeared to have little effect on normal hematopoietic cells. Electroporation causes transient pore formation in membranes, and with optimisation can get 100% transfection and suppression of c-myc and decreased cell viability. In control cells plus antisense the oligo is sequestred to the cytoplasmic vesicles. However with electroporation the oligo localises to the nucleus and non-vesicular cytoplasmic structures in cytoplasm. No effect of c-myc oligos were seen without electroporation. This method allows up to a 7-fold increase in signal inside cells following electroporation (Bergan et.al.1996).

Non-specific (polyanionic effects) of antisense

Many so-called sequence-dependent antisense-specific effects of phosphorothioate oligodeoxynucleotides can potentially be explained by the non-specific targetting of phosphorothioates to cell adhesion molecules. Here they may act as agonists or antagonists of natural ligands depending on a) antisense sequence or b) the target cell phenotype.

This study and others (Zhao et.al.1996), have shown preferential uptake of polyanionic molecules such as heparins, or antisense phosphorothioate molecules to myeloid cells. Heparin (a linear, sulphated, negatively charged polysaccharide) binding is found to be higher in human granulocytes (264-2458 fluorescence units-FU) than in monocytes (10-80 FU) and lymphocytes (5-31FU). It has been described as possessing anti-inflammatory, anti-metastatic and anti-atherosclerotic properties (Bleiberg et.al.1981).

In the study described in this thesis antisense binding shows a clear delineation of myeloid > B cells > T cells (CD4=CD8). The following decreasing levels of uptake: peripheral blood CD33 > CD22 > CD8 = CD4: bone marrow CD15 > CD20 > CD7 (Zhao et.al.1996). Similarly in acute myeloid leukaemia CD34+ leukemic blasts were able to take up more antisense than normal CD34+ cells, an effect potentiated by IL-3 and SCF. Normal CD34+ bone marrow cells have a relatively low uptake unless stimulated by IL-3 and IL-6. Similarly heparin (0.1-100μg/ml) was able to inhibit the growth of U-937 leukaemic cell proliferation and is also able to induce terminal differentiation (Volpi et.al.1994) in these cells. This effect is related to charge density, particularly the degree of N-sulphation (of glucosamine) which is also required for heparin binding to the complement C3Receptor (CD11b-CD18) (Diamond et.al.1995).

It seems likely that the higher levels of intracellular uptake of antisense into monocytes and neutrophils are a direct consequence of higher levels of extracellular binding to these cells. Higher membrane associated antisense on monocytes and neutrophils may represent higher levels of DNA-binding proteins. This may be related to the levels of CD11b and other, similar heparin-binding proteins on the cell surface. Extracellular levels of antisense on monocytes and neutrophils were found to decrease over time. Therefore it is reasonable to consider that this decrease may represent a down-regulation of these binding proteins or receptors despite the continued presence of the oligonucleotide. This may occur either as a consequence of DNA binding and subsequent internalisation, or changes in affinity or cell phenotype during culture which is known to occur when CD11b is activated.

Phagocytic cells such as monocytes and neutrophils posess storage pools of β2 integrins (notably Mac-1) in peroxidase-negative granules and are able to increase levels up to ten fold in response to pro-inflammatory mediators (Smyth et.al.1993). Heparin is also able to up-regulate the degree of CD11b expression, an effect which mimics TNF-α, G-CSF, arachidonic acid, or IL-8 treated neutrophils (Diamond et.al.1995; Ohsaka et.al.1997). Heparin binds both Mac-1 (CD11b-CD18) and CD45 (Coombe et.al.1994) and blocks their binding to stroma, an effect which is dependent on the degree of heparin sulphation. However only certain isoforms of CD45 (the 180 kDa lacking the three terminal exons) are able to bind glycosaminoglycans. CD45 is able to regulate myeloid cell proliferation in association with IL-3 and GM-CSF.

Fibrinogen, a homodimeric 340kDa glycoprotein, which is a natural ligand for Mac-1 binds the RGDS (Arg-Gly-Asp-Ser) sequence, which is also recognised by fibronectin, vitronectin, von Willebrand factor, the tat (transactivation) protein of HIV-1 and other molecules (Levesque et.al.1991). Fibrinogen binds other extracellular-matrix components and also proteases of the coagulation cascade. It also promotes adhesion and motility to endothelial cells through the vitronectin receptor. Fibrinogen interacts with activated complement receptors CR3 (αmβ2) and CR4 (αxβ2) on monocytes, macrophages and neutrophils. Fibrinogen has also been found to be mitogenic for human hematopoietic progenitors and increases CFU-GM formation in Dexter-type long-term bone-marrow cultures. Fibrinogen is able to enhance the mitogenic effect of IL-3 on CD34+ progenitors in CFU-GEMM assay, an effect which is not inhibited by RGD peptides (Levesque et.al.1991). CD34 is expressed on hematopoietic progenitors, high endothelial venules (HEV), vascular endothelium and some fibroblasts. It is a mucin-like sialoprotein which contains a large number of O-linked and N-linked glycosylation sites. It may play a role in haematopoietic progenitor cell adhesion to bone marrow stroma through L-selectin binding (Carlos et.al.1994).

Therefore it is likely that antisense molecules may act as partial agonists or antagonsists for heparin or fibrinogen-type interactions depending on the individual cell type and antisense sequence. Agonists of the fibrinogen receptor (αiibβ3) increase its binding properties and cause poly-phosphoinositide hydrolysis and release of diacylglycerol and IP3 (inositol triphosphate) which activate protein kinase C (Smyth et.al.1993).

Phorbol esters such as phorbol myristyl-acetate (PMA) are able to differentiate HL-60 promyelocytic leukaemia cells also cause an increase in β3 subunit phosphorylation. Agonists which promote β2 integrin activation cause sub-unit phosphorylation, increase intracellular Ca2+ and stimulate protein kinase C activity (Smyth et.al.1993). Loading neutrophils with thiophosphate (a non-hydrolysable phosphate) enhances αm β2 activity and prevents deactivation. Agonists of αm β2 regulate neutrophil phospholipases and stimulate phospholipase A2. Agonists however, which elevate

cAMP or lower intra-cellular Ca2+ however promote inactivation of β2 integrins, high cAMP levels can also inhibit smooth muscle growth (Indolfi et.al.1996).

Pro-inflammatory mediators promote the binding of neutrophils and monocytes to fibrinogen, factor X, C3bi, by activating β2 integrins Mac-1 (αm β2) and p150,95. Activation of β2 integrins peak within 5-10 mins of stimulation and decreases within 30 minutes. This mimics the findings described above with antisense binding to monocytes and neutrophils. Mac-1 activation results in a rapid increase in avidity followed by recruitment of further receptors from the cytoplasmic granule store (Zimmerman et.al.1992). Surface expression of Mac-1 can be increased by a variety of agonists including calcium ionophores, phorbol esters, fMetLeuPhe, GM-CSF, C5a, TNF-α, LTB4. Mac-1 ligands include ICAM-1, fibrinogen, factor X, complement C3bi.

Neutrophil adhesion depends on CD11b-CD18 and CD11a-CD18 interactions with endothelium and anti-CD18 antibodies can inhibit neutrophil migration to the lungs. Stimuli which up-regulate Mac-1 also down-regulate L-selectin (Carlos et.al.1994), β2 integrins are therefore important in neutrophil, monocyte and lymphocyte transmigration across enothelium. P and L selectins also bind heparin, sulphatides and sulphated polysaccharides. CD34, a ligand for L-selectin present on high endothelial venules (HEV) in murine peripheral lymphoid tissue and hematopoietic progenitor cells (Carlos et.al.1994) might therefore also be inhibited by antisense phosphorothioate molecules. Platelet aggregating factor (PAF), synthesised by endothelial cells in response to thrombin, histamine or LTB4 (Zimmerman et.al.1992) binds to its receptor on neutrophils and up-regulates CD11b-CD18 expression.

There are many other potential non-specific immunomodulatory properties of antisense molecules. Phosphorothioates bind CD4 in a length-dependent manner, and will also bind to basic FGF, acid FGF, VEGF, PDGF (Stein, 1995). Phosphorothioate antisense to c-myb used to prevent re-stenosis following angioplasty appear to mediate their effect through inhibition of heparin-binding growth factors (Wang et.al.1997). Phosphorothioate antisense molecules have also been shown to bind bFGF receptors with a high affinity (Benimetskaya et.al.1997). Antibodies which inhibit CD11b-CD18 also prevent reperfusion injury (Arnaout, 1993). Phosphorothioates also bind laminin and prevent adhesion, an effect which is dependent on the presence of the G-quartet and flanking sequences. CpG is an immunomodulatory sequence, and phosphorothioates are able to cause an increase in polyclonal immunoglobulin secretion and expression of class I and II MHC (Stein, 1995).

High levels of dGMP and dAMP from breakdown of oligodeoxynucleotides can have varied and pronounced effects on haemopoietic tissue (Milligan et.al.1994). Short sequences within oligodeoxynucleotides may have anti-proliferative effects, therefore scrambled or sense sequences by design will not have these effects eg. the G-4 sequence (so-called G-quartet) has anti-proliferative effects. Heparins also have anti-proliferative effects (as does dextran sulphate (Volpi et.al.1994; Morahan et.al.1972; Regelson, 1968). Oligodeoxynucleotides can act as protein kinase c inhibitors and a phosphorothioate homopolymer of dC-28 has been shown to inhibit HIV infection of CD4+ cells (Milligan et.al.1994). Some of these non-specific effects may be however reduced by using low (1-5μM) concentrations of oligodeoxynucleotides and short (15-17 bases) sequences (Stein, 1995).

In B cell lymphoma where results of a recent antisense bcl-2 clinical trial have shown promising results (Webb et.al.1997) data might be explained by antisense effects on polyclonal B cell activation through binding CR2 (CD21) leading to apoptosis of B cells. This is supported by findings from others who showed a progressive antisense uptake in more mature B cells, where the order of uptake was pre B> pro B> pre-pro B cells > Thy1+ cells (Zhao et.al.1996; Zhao et.al.1994). Dextran sulphate had a similar cellular localisation to phosphorothioate antisense, again demonstrating a non-specific uptake mechanism for polyanions.

Given the association of antisense phosphorothioate molecules with CD11b and their subsequent internalisation into the endosomal compartment, strategies involving lysosomal disruption have been attempted. Monesin has been shown to disrupt endosomal pH by reduceing the acid pH and the pH-dependent quenching of FITC. 100μM chloroquine reduces ligand delivery to lysosomes during transfection and it was found to be able to increase the efficiency of delivery of DNA into liver cells (Erbacher et.al.1996). However ammonium chloride, spermine, methylamine (all of which similarly affect lysosomal pH) had no effect. Chloroquine accumulates in these vesicles and can reach levels of 9mM in cells (100μM, 4hrs) radically elevating lysosomal pH.

Other methods of enhancing DNA transfection have similarly been attempted to enhance the bio-availibility of antisense: cyclodextrins (Zhao et.al.1995); liposome encapsulation (Farhood et.al.1994); cholesterol conjugation (Zhao et.al.1995); poly-L-lysine conjugates (Zhao et.al.1995; Wagner et.al.1990); Streptolysin O reversible cell permeabilisation was demonstrated to achieve up to a 100 fold increase in delivery of DNA to the nucleus (Spiller et.al.1995). All of these strategies whilst interesting research tools still have some way to go before being adopted as routine therapeutic strategies.

Yet other studies have concentrated on backbone modifications to enhance stability. Chimeric molecules of methylphosphonate-phosphodiester antisense analogues have been shown to have a higher selectivity. Phosphorothioates may target RNAse-H activity against partial complementary sequences of cDNA-RNA. Using chimeric methylphosphonate-phosphodiester antisense the ranking of efficiency on ability to inhibit bcr-abl mRNA is methylphosphonate-phosphodiester > phosphodiester >=phosphorothioate > methylphosphonate-phosphorothioate (Giles et.al.1995). Adding a 5'-propyne group increases the affinity of antisense for target sequences (Tonkinson et.al.1994) and retains RNAse-H activity, while reducing the size and using tandem oligodeoxynucleotides eg. 2 x 7mers to the target sequence may allow synergistic action, and also help reduce non-specific or polyanionic effects caused by longer sequences (Woolf, 1996).

Other targets in AML

In the treatment of acute leukaemia the primary target for therapy has been the highly replicative leukaemic blast cell. Mostly these cells are sensitive to S-phase specific drugs and conventional chemotherapy has achieved largely succesful treatment of these cells. However, as previously described there is frequent disease relapse in AML. This may be due to the presence of persistant residual, slowly-replicative, leukaemic "stem cells" which are insensitive to S-phase specific drugs (Terpstra et.al.1997). These are the cells which are most likely to give rise to drug-resistant daughter cells and to contribute to disease relapse. This presents a far more difficult target for therapy since these cells are to all intents very similar to normal haematopoietic stem cells. It is these cells however which must be identified and then targetted to provide a long-term treatment option for patients with refractory or resistant disease.

The recent study described by (Terpstra et.al.1996) has found the CD34 negative fraction of AML cells contained both immature and mature cobblestone-area forming cells (CAFC) and produced high numbers of CFU-AML in long-term *in vitro* bone-marrow cultures (LT-BMC) . The CD34 positive fraction however only produced small numbers of CFU-AML, mature CAFC, early cobblestone-forming areas (equivalent to haematopoietic progenitors) and late cobblestone-forming areas (equivalent to stem cell progeny). For engraftment into SCID (severe combined immunodeficiency) mice required 7.5 x 10^6 cells for engraftment at day 35, however transplantation of 10^6 cells took between 55-106 days for engraftment. The CFU-AML were mostly recovered in the CD34 negative fraction, whereas few were recovered in the CD34+ fraction. Early cobblestone forming areas were found in the CD34 negative fraction and late cobblestone areas in both CD34 positive and negative populations. Late CAFC in humans are normally CD34+, Rhodamine 123 ^{dull}, HLA-DR+^{low}. Both CD34+ and CD34 negative cells were able to repopulate SCID mice. Bonnet and Dick (1997) however found that SCID long-term-initiating-culture cells were exclusively CD34+38- cells similar to the normal SCID repopulating cells.

This therefore gave rise to two possible theories for leukaemic development. In the first model the leukaemic process blocks normal development and the subsequent degree of target cell commitment influences the stage of the leukaemic blasts. The second model suggests that mutations arise in more primitive cells and the blast cell heterogeneity results from variability in the ability to differentiate. This theory predicts little variability in the phenotype of leukaemic stem cells from different patients.

Most AML blasts have limited proliferative capacity and therefore must be constantly replenished by a limited number of more primitive cells. Only 10-20% of CD34+ cells

proliferate *in vitro* to give rise to colonies and approximately one cell in 2 x 10^5 cells is able to repopulate SCID mice (Bonnet et.al.1997). CFU-L treated with 5-FU from AML patients were transplanted into SCID mice and were able to re-establish the original AML classification in the mice (Terpstra et.al.1997) again indicating the leukaemia-initiating cells were quiescent during therapy. These CFU-AML could be replated to form secondary colonies and is consistent with AML being a progenitor cell disease. Therefore it is the levels of these slowly dividing CFU-L which probably contributes to the level of disease resistance to S-phase-specific chemotherapy and subsequent relapse.

Other markers, such as the myeloid progenitor-specific galactose-binding residue found on approximately 8% of CD34+ cells (Pipia et.al.1997), or Rhodamine ^{dull} cells, in combination with CD38 or CD34 staining may be required to discriminate leukaemic from normal haematopoietic progenitor cells. CD34 positive selection of cells for autologous transplantation in AML may therefore contribute to disease relapse in patients with CD34 positive AML progenitors whereas CD34 depletion would obviously remove normal progenitors. CD34 positive selection in combination with CD15 depletion has been shown to provide a reliable reduction in leukaemic progenitors (Rubin et.al.1994) without depleting normal progenitors.

However until reliable data confirming the presence or absence of a particular cell marker becomes available to identify leukaemic progenitors purging of bone marrow and leukapheresis products will remain ineffective and controversial in the clinical setting. Similarly given that studies have demonstrated only a contribution to disease relapse from transplanted leukaemic cells, albeit at a very low level (Rill et.al.1994; Brenner et.al.1993), it therefore remains that the most likely source of tumour cell contamination, and hence disease relapse, arises from resistant and presumably non-cycling leukaemic progenitors present in the bone marrow and peripheral circulation of the patient themselves.

Treatment to destroy leukaemic progenitors in AML is unlikely to be completely effective given the low proliferative index of these cells (Terpstra et.al.1997). Long-term remission may be achieved if the transplanted tumour load is sufficiently low to allow an effective haematopoietic recovery (Dwenger et.al.1996). However, if as indicated above, only a percentage of leukaemic cells are capable of re-establishing the original disease (Bonnet et.al.1997) then it is likely only a small contribution to relapse can arise from the transplanted tumour cells. A more effective form of cytotoxic therapy must therefore be found to eradicate these slowly proliferating cells. Either cells must be mobilised into cycle using growth-factors or cycle-independent cytotoxic agents must be chosen. Therefore at present the only treatment to effect a cure lies with

allogeneic transplantation with a limited graft-versus-host (and therefore graft-versus-leukaemia) response to destroy residual tumour cells.

Cell cycle control in AML

The amounts of growth factor required for maintenance of cellular viability are much lower than are required for proliferation or for functional cellular activation, and if progenitors are not in cycle, then appropriate CSF may be able to induce S-phase in a number of hours (Nicola, 1989). The decision to exit a self-renewing proliferative pathway and enter the differentiation-commitment pathway appears to occur in the G1 phase of the cell cycle. Constitutively activated growth-factor genes eg. GM-CSF do not cause leukaemic transformation on their own but may be a secondary effect in leukaemic development (Lang et.al.1990). Differentiation and proliferation may be therefore uncoupled in leukaemia. Growth factor receptors may contain separate intracytoplasmic domains for each function and the coupling of growth and transcription factors may control proliferation, differentiation and survival of myeloid cells. Cell cycle regulation has an important role in this coupling. Duration of G1 influences differentiation, with strong proliferative signals from some cytokines shortening G1 and suppressing differentiation (Olsson et.al.1996).

Hypophosphorylation of the retinoblastoma protein for example is also associated with differentiation. Similarly down-regulation of cdk4 levels (whose primary substrate is the retinoblastoma protein) induces differentiation into granulocytes. Overexpression of cyclin D2 shortens G1 and decreases differentiation. DNA damage detected by p53 induces p21waf and inhibits CDK which arrests cells in G1 for DNA repair or apoptosis. Autonomously growing AML blasts have been shown to lack the retinoblastoma protein (Olsson et.al.1996).

The initial phase of G1 is strictly dependent on mitogenic stimuli, however as cells progress through the restriction (R) point there is a much reduced dependence on growth factors. Control of the restriction point allows cells to withdraw into quiescence (G0), repair DNA or differentiate. D-type cyclins seem to be the R point proteins, with the retinoblastoma protein (Rb) as the major negative regulator and cyclin dependent kinases as additional components (cdk4 and cdk6 and their inhibitors cki). Rb sequestrates transcription factors required for progress through S phase and is regulated by phosphorylation (which inactivates its repression of cycle). Neutralisation of cyclin D0 by antibody prevents phosphorylation of Rb *in vivo* and arrests cycling cells in late G1(Strauss et.al.1995).

Overexpression of D-type cyclins has been shown to shorten G1 and reduces the requirement for exogenous growth factors and can prevent terminal differentiation.

Exit from cell cycle can facilitate terminal differentiation whereas tumour cells abandon control mechanisms and remain in cycle without maturing. This is obviously a feature of many myeloid leukaemias and remains to be investigated. Cyclin D0 (coded on chromosome 11q13), and cyclin-dependent kinases (cdk) and cki counterparts are commonly de-regulated in other cancers (Strauss et.al.1995). These D-type cyclins act as growth factor sensors and their expression depends on extracellular cues rather than cell-cycle status. Mitogen withdrawal leads to cessation of D-type cyclin synthesis and cells rapidly exit cycle. Inhibitors of cdk4 and cdk 6 block cyclin D dependent kinases and cause G1 arrest ie. cells do not enter S phase. In cells without retinoblastoma protein (in many cases of AML) these inhibitors do not cause G1 arrest. Retinoblastoma regulates the E2F transcription factor family by sequestering them when Rb is hypophosphorylated whereas phosphorylation releases these transcription factors and allows transcription. Inactivation of Rb reduces cellular requirement for mitogens. When cells enter S-phase cyclin E and E2F are inactivated. Cyclin D0 is overexpressed in many cancers, though p16ink4α loss may mimic this D0 overexpression. p53 mediates the CDK inhibition of p21cipi and is partially responsible for G1 arrest, though loss of retinoblastoma can bypass p53 mediated G1 arrest. Most proto-oncogenes mimic persistent mitogenic stimuli whose pathways converge to mediate cellular passage through G1 (Sherr, 1996).

Therefore manipulation of cyclin activity may be an alternative target in leukaemic therapy whether through antisense mechanisms or secondary messenger stimulation. Growth factors, in addition to regulating cyclin activity also regulate bcl-2 levels. Perhaps a convergent pathway also exists which allows simultaneous cessation of cell cycling whilst reducing the levels of bcl-2 which are required to survive this period of quiescence. It may be that the findings outlined in this thesis the findings that antisense bcl-2 in combination with chemotherapeutic agents is more effective than cytosine arabinoside on its own the mitogenic capacity is disrupted through p53 or retinoblastoma-mediated mechanisms whilst antisense bcl-2 reduces the cell's capacity for survival in the absence of regulatory signals such as cyclins which may help explain why antisense on its own has little effect. In cells treated with antisense alone there is no pro-apoptotic stimulus to provide activation of cytochrome c or AIF release from the mitochondria therefore reduction in bcl-2 protein levels should be expected to return to normal after removal of the antisense.

Therapy

Autocrine stimulation alone is not sufficient for leukaemic development but may considerably enhance the proliferative capacity of these cells. For example most myelomonocytic leukaemias express receptors for GM-CSF, however growth factors and their receptors may also interact in an intracellular compartment and not be detectable on the cell surface. It may be the differentiated, non-proliferative progeny of these cells which secrete growth factors causing stimulation of the blast cells (Lang et.al.1990).

Treatment of AML with recombinant growth factors to increase the percentage of cycling S-phase blasts as an addition to chemotherapy is a potiential treatment option but will probably require considerable monitoring of blast behaviour *in vitro* on a patient-to-patient basis. GM-CSF treatment *in vivo* in 96% AML patients has been shown to increase the percentage of leukaemic blasts in S-phase, and there is a clonal expansion of blasts with the greatest increase in myeloblasts was seen in AML M6 patients (Baer et.al. 1996). G-CSF receptors have been shown to be present in up to 97% of AML patients and treatment with G-CSF caused terminal differentiation of some leukaemic cells into neutrophils *in vitro* (Bernstein, 1993). Administration of G-CSF after chemotherapy has not been shown to have a higher incidence of resistant disease or relapse (Baer et.al. 1996). However normal CFU-GM are also sensitive to cytosine arabinoside at 10^{-6}M (the clinical plasma levels are approximately 10^{-5}M) an effect which is sensitised by the administration of G+GM-CSF or IL-3. Cytosine arabinoside at 10^{-8}M in the presence of stem cell factor (SCF) plus G and GM-CSF destroyed blast cell progenitors by recruiting more blasts into S-phase (Smith et.al.1996).

Differentiation therapy

There is also the potential for differentiation of leukaemic blasts either by growth factors or pharmaceutical methods. HL-60 cells treated with 10ng/ml phorbol myristate acetate (PMA) for 2 days causes cytostasis and differentiation measured by up-regulation of CD11b and CD14 expression, implicating protein kinase c signalling pathways in monocytic differentiation. Bcl-2 mRNA was rapidly down-regulated by this treatment while bax and bcl-xl mRNA were not altered. Bcl-2 mRNA down regulation was seen only in the parental cell and not in the adriamycin-resistant form even though both forms were able to differentiate. This suggests that bcl-2 protein down-regulation is not required for differentiation. Bcl-2 protein half-life was also increased in the drug-resistant form (Blagosklonny et.al.1996). Similarly U937 cells have low levels of bcl-2 but when transfected with a plasmid containing bcl-2 they

increased their resistance to cytosine arabinoside by 3 logs (Suresh et.al.1996). Arsenic trioxide is an effective component of some traditional Chinese herbal treatment for APML even in patients resistant to ATRA. ATRA treatment causes bcl-2 protein and mRNA are down-regulated and apoptosis occurs independent of retinoic acid. HL-60 cells however were unaffected by arsenic treatment nor were U937 cells. Arsenic is thought to interfere with phosphorylation-dephosphorylation reactions by replacing phosphate groups and is also able to bind sulphydryl (-SH) groups (Chen et.al.1996) and presumably modifies the phosphorylation status of important proteins required for induction of apoptosis.

Thus this data serves to support the hyopothesis that antisense-mediated effects in areas as diverse as cancer therapy, immune-modulation, and pro-inflammatory disease will require explicit controls in order to exclude the possiblity of non-antisense, sequence-specific effects. Intracellular calcium, phospholipase A2, protein kinase C, polyanionic and integrin-mediated effects effects will all have to be controlled for and excluded in order to justify some of the more extravagant claims on behalf of antisense molecules. The temptation to see simple cause-and-effect relationships is real but may obscure some of the more interesting effects mediated by antisense molecules. Future studies could test this by pre-incubating cells in the absence and presence of unlabelled antisense, prior to the addition of the labelled molecule or an unlabelled ligand for specific receptors.

This information might also prove clinically useful, since one could envisage pre-treatment of patients with a short-lived non-therapeutic oligonucleotide to reduce non-specific plasma clearance, extracellular binding, and subsequent sequestration, of therapeutic oligonucleotide to sites other than the target. This may also be achieved with saturation with synthetic heparin analogues which have no therapeutic effect (Harenberg et.al.1995; Diamond et.al.1995; Bleiberg et.al.1981). It must also be noted that all experiments perfomed in the present study were conducted using isolated cells. However, in the clinical situation, there may be a variety of factors, such as plasma protein binding, which may reduce the bio-availability of antisense to the cells as already seen in the much reduced levels of antisense uptake in the presence of serum. Thus the therapeutic plasma levels required may be far in excess of those studied here.

There is however a positive outcome from this study in that cells which express CD11b have higher levels of antisense than those which do not. Fortuitously AML cells with CD11b expression (a recently described novel sub-set of AML) which have a poorer complete remission response (Paietta et.al 1998) are more likely to internalise antisense phosphorothioate molecules. These cells are more likely to have elevated bcl-2 expression and grow autonomously and are therefore more difficult to treat with standard chemotherapy. Antisense bcl-2 (or other targets as described above) may therefore tip the balance of survival in these cells, when treated with standard chemotherapy, towards apoptosis. This may be envisaged in either a purging setting or in the elimination of residual disease where more dramatic effects are likely to be seen where the potential for CFU-AML to contaminate autologous transplants is reduced at the outset. It is likely to be more successful, that rather than looking for (then removing from) the proverbial needle in the haystack, to ensure that it never gets there in the first place.

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